EXPERIMENTAL CELL RESEARCH

UNIVERSITY OF HAWAII
JUN 30 '50

Published under the auspices of

THE INTERNATIONAL SOCIETY FOR CELL BIOLOGY

EDITORS:

TORBJÖRN CASPERSSON · HONOR B. FELL · JOHN RUNNSTRÖM

FRANCIS O. SCHMITT · PAUL WEISS · RALPH W. G. WYCKOFF

VOLUME 1 · APRIL 1950 · NUMBER 2

ACADEMIC PRESS INC., PUBLISHERS, NEW YORK, N.Y.

EXPERIMENTAL CELL RESEARCH

EDITORS:

T. Caspersson, Department of Cell Research, Karolinska Inst., Stockholm 60, Sweden
Honor B. Fell, Strangeways Laboratory, Cambridge, England
J. Runnström, The Wenner-Gren Institute for Experimental Biology, Stockholm, Sweden
Francis O. Schmitt, Massachusetts Institute of Technology, Cambridge 39, Massachusetts
Paul Weiss, University of Chicago, Chicago 37, Illinois
Ralph W. G. Wyckoff, Laboratory of Physical Biology, National Institute of Health, Bethesda, Maryland.

EXECUTIVE COMMITTEE:

T. Caspersson, Honor B. Fell, J. Runnström and J. F. Danielli, King's College, London, W. C. 2, England.

ADVISORY BOARD:

H. Bauer (Wilhelmshafen), W. Bloom (Chicago), J. Brachet (Bruxelles), F. Buchthal (Copenhagen), A. Buzzati-Traverso (Verbania Pallanza), R. Chambers (New York), E. Chargaff (New York), J. F. Danielli (London), C. D. Darlington (London), J. N. Davidson (Glasgow), M. Demerec (Cold Spring Harbour, N. Y.), J. Engelbreth-Holm (Copenhagen), E. Fauré-Frémiet (Paris), A. Fischer (Copenhagen), A. Frey-Wyssling (Zürich), N. Fries (Uppsala), R. J. Gautheret (Paris), L. Geitler (Wien), G. Gomori (Chicago), A. Haddow (London), E. Hammarsten (Stockholm), B. Hanström (Lund), E. Newton Harvey (Princeton, N. J.), L. V. Heilbrunn (Philadelphia, Pa), G. C. Heringa (Amsterdam), A. Hollaender (Oak Ridge, Tenn.), H. Holmgren (Stockholm), H. Holter (Copenhagen), J. Holtfreter (Rochester, N. Y.), P. Koller (London), F. Lehmann (Bern), P. L'Héritier (Paris), G. Levi (Torino), P. E. Lindahl (Uppsala), G. Lindeberg (Uppsala), C. C. Lindegren (Carbondale, Ill.), R. J. Ludford (London), S. E. Luria (Bloomington, Ind.), A. Lwoff (Paris), A. Mirsky (New York), G. Montalenti (Naples), H. Okkels (Copenhagen), O. Olivo (Bologna), A. W. Pollister (New York), J. Randall (London), W. J. Schmidt (Giessen), F. Schrader (New York), W. Seifriz (Philadelphia, Pa), T. M. Sonneborn (Bloomington, Ind.), J. M. Sosa (Montevideo), J. Spek (Rostock), W. M. Stanley (Berkeley, Calif.), E. Suomalainen (Helsingfors), E. L. Tatum (Stanford University, Calif.), T. Teorell (Uppsala), A. Tyler (Pasadena, Calif.), W. Wilbrandt (Bern).

Subscriptions

EXPERIMENTAL CELL RESEARCH

will be published in four issues annually at $10.00 per volume. The price of one issue is $3.00. Please send your subscription orders to Academic Press Inc., 125 East 23 St., New York 10, N.Y.

THE QUANTITATIVE ESTIMATION OF DESOXYRIBOSENUCLEIC ACID BASED ON METHYL GREEN STAINING

N. B. KURNICK, M. D.[1]

Department of Cell Research, Karolinska Institutet, Stockholm

Received June 30, 1949

Investigations performed by the author (3, 4, 5) in 1947 and the spring of 1948 at the Rockefeller Institute for Medical Research, New York, demonstrated that staining of nucleic acids with methyl green and pyronin was dependent upon the state of polymerization of the substrate. Whereas highly polymerized desoxyribonucleic acid (DNA) stained with methyl green in the ratio of 1 molecule dye to 10 atoms of P, the same acid upon depolymerization by the specific enzyme or heat (60°C for 10 minutes is sufficient) loses its capacity to stain with methyl green and stains instead with pyronin (irrespective of whether the stains are applied in a mixture or alone). These experiments were carried out with extracted, purified nucleic acids and nucleoproteins and also with isolated calf thymus nuclei prepared by a modification of Stoneburg's method (10). In an extension of the work on the effect of heat on methyl green staining, Pollister and Leuchtenberger (9) showed that the loss of methyl green staining by chromatin in mouse liver sections following heating is not associated with loss of nucleic acid phosphorus, U.V. absorption, or Feulgen staining, thus confirming the role of polymerization in determining staining with methyl green.

The observation that, with suitable control of pH, methyl green stained polymerized DNA in the constant ratio of 1 molecule dye to 10 atoms phosphorus, made it appear probable that a quantitative histochemical technique could be developed, based on this dye. Our earlier studies (4) had shown that nuclei isolated in bulk, bound the expected amount of methyl green after they had been freed of histone by dilute HCl. Histone interfered with the dye combination so that approximately 50 per cent of the expected values were obtained if histone was not removed. It was also found that

[1] This work was done under a fellowship awarded by the American Cancer Society on the recommendation of the Committee on Growth of National Research Council. Permanent address: Department of Medicine, Tulane University School of Medicine, New Orleans, Louisiana U. S. A.

methyl green was very unstable at pH's outside the range 3.5–5.0, and that the loss of color was accelerated by exposure to light at pH's outside this range. The following method was therefore adopted.

METHOD

A. *Staining and photometry*

The methyl green, Cert. N.G. 26 (ethylated hexamethyl pararosaniline), National Aniline Division, "85 per cent dye content" (found 54 per cent (4)) C.I. 685, was dissolved in 0.2M pH 4.1 acetate buffer to make a 0.25 per cent by weight (of the commercial dye) solution. This solution was extracted repeatedly with $CHCl_3$ to remove crystal violet until no further color was removed by the $CHCl_3$. The dye solution was stored in dark bottles in the dark under which conditions it was stable for many months. The molar extinction coefficient at its maximum, 630 mμ, is 74,400 (4).

The preparation to be stained is immersed for 5 minutes in 0.1 N HCl, rinsed in water, placed for 5–10 minutes in 0.2 M pH 4.1 acetate buffer, transferred to the dye solution and allowed to stand overnight at 4°C (refrigerator). The slide is then rinsed in two changes of 0.05 M pH 4.1 acetate buffer for 10 minutes each, covered with a cover glass and sealed with paraffin. Alternatively, the slides were transferred from the second buffer rinse to a mixture of 4 parts glycerin and 1 part 0.2 M acetate buffer, and mounted in this. It was not found possible to dehydrate the stained material with alcohol for permanent mounting because the stain faded rapidly on exposure to light after such treatment. The dye content was measured microphotometrically in the apparatus described by Caspersson (1) at 645 mμ, using 90 × glass apochromatic objective, 5 × quartz ocular, ocular to photomultiplier tube about 3 meters.

Photomultiplier tubes were used and the deflections read on a multiflex galvanometer (used as a null-point instrument by adjustment of the variable rotating sector). The photo-tube aperture admits an illuminated circle 3–5 μ in diameter as measured at the object plane. The light source was a tungsten band projector lamp focused with a convex lens of long focal length on the entrance slit of the monochromator.

B. *Preparation of Material*

Most tissues handled by the usual histological techniques, yield nuclei which stain heterogeneously in that they contain blocks and strands of chromatin, unstained regions, and nucleoli. Such structures are unsuitable

for quantitative photometric analysis. The immersion of the tissue in 30 per cent sucrose (2) before fixation yields nuclei which are homogeneous except for the nucleoli. Fixation in *neutral* formalin preserves this structure.

1. *Calf thymus.* Fresh calf thymus was teased with dissecting needles in 30 per cent sucrose solution, let stand at 4°C for about 15 minutes, smeared on glass slides and transferred to 4 per cent neutral formaldehyde solution for several hours to overnight. Homogeneous, structureless nuclei result which can be stained as described above. The nuclei stain a uniform, clear, blue-green. The occasional non-homogeneous nuclei which remain, stain a dirty, greenish brown. It was also found possible to transfer the suspension of thymocytes in 30 per cent sucrose to neutral formalin in a test-tube. This fixed suspension could then be stored in the refrigerator, dried onto glass slides at room temperature, and stained as in A when required.

2. *Mouse Liver.* Small pieces of fresh liver were suspended in the cold for two hours in 30 per cent sucrose, transferred to 4 per cent neutral formaldehyde overnight, embedded in paraffin and sectioned in the usual manner at 4 μ, taking care that the temperature did not exceed 54°C during embedding. Many of the nuclei, as determined by hematoxylin-eosin stain and photography with U.V. light at 257 mμ are homogeneous except for the nucleoli.

3. *Chicken erythrocytes.* Chicken blood from the comb was smeared on glass slides and allowed to dry at room temperature. When these were treated as in A, all the nuclei were found to be homogeneous, clear blue-green. If the HCl treatment was omitted, the nuclei revealed chromatin blocks with empty spaces between them except for a rare homogeneous nucleus which stained only very slightly.

RESULTS

The results of a number of analyses are listed in the table. For whole cells, the DNA content was calculated on the basis of the formula:

$$\text{DNA in mg} = \left(E_{645} - \frac{550^4}{645^4} \times E_{550}\right) \times \text{Area in sq. cm} \times 310 \times 10 \times \frac{1}{75{,}000},$$

where the factor 310 corresponds to the atomic weight of P multiplied by 10 for the phosphorus content of DNA, 10 to the number of atoms of P per molecule of dye, and 75,000 to the molar extinction coefficient of methyl green.[1] Whereas methyl green combined with DNA in solution reveals an

[1] The phrase in parentheses represents the E corrected for non-specific absorption by the application of Rayleigh's law to the measured absorption at 550 mμ where methyl green has practically no absorption. The correction is usually negligible.

TABLE I

Material treated as in A unless otherwise indicated	E_{645}	E_{630}	E_{550}	E_{700}	Diameters in μ	DNA Content $\times 10^{-8}$mg
Calf thymus	.342	.288	.050	.070	8.4	0.7
Calf thymus	.364	.352	.097	.054	7.5	0.6
Calf thymus	.366	.269	.062	.059	8.8	0.8
Calf thymus	.284	.262	.076	.047	8.9	0.6
Calf thymus	.413	.350	.070		8.0	0.7
Calf thymus, HCl omitted	.259	.192	.052		5.6	0.2
Calf thymus, HCl omitted	.242	.265	.132		7.5	0.3
Calf thymus, sucrose omitted	.290	.323	.292	.108	9.4	0.4
Mouse liver, 4 μ section	.138	.128	.068	.012	10.8	0.7[1]
Mouse liver, 4 μ section, embedded at 60°–65°C	.094	.100	.119		10.8	0.21[1]
Chicken RBC	.121	.093	.021	.004	6.6 × 7.0	0.17
Chicken RBC	.119	.119	.046	0	7.0 × 7.5	0.16
Chicken RBC	.137	119	.018	.002	7.5 × 5.6	0.17
Chicken RBC	.149	.130	.035	0	7.5 × 5.6	0.18
Chicken RBC, HCl omitted	.090	.086	.030		6.1 × 5.2	0.08
Unstained chicken RBC	.008		.020			
	.017		.019			
Unstained mouse liver	.034		.073			
	.042		.062			

[1] Calculated for sphere 10.8 μ in diameter.

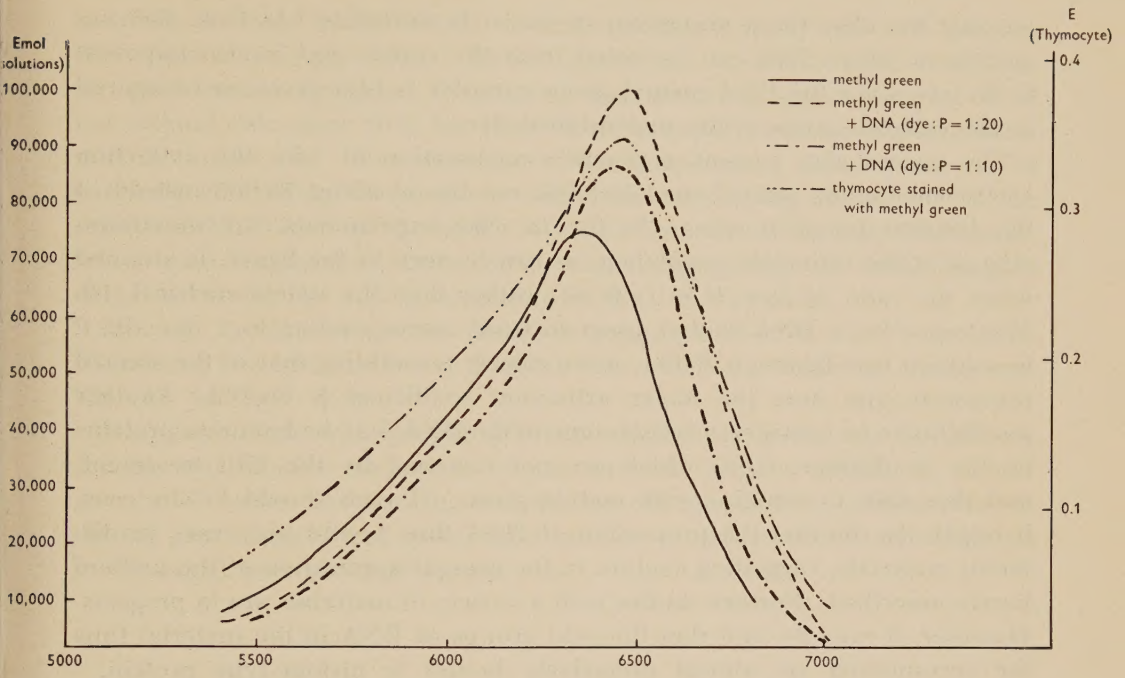
increase in the molar extinction coefficient to 100,000 at the new maximum of 645 mμ from 74,400 at the former maximum of 630 mμ (4), 75,000 is used in these calculations for reasons given in the discussion. Since so large an area of the cell surface (3–5 μ diameter) is included in the photocell aperture and since drying the cell onto the glass slides causes it to become attached by a broad base, the nuclei are regarded as cylinders instead of spheres. This permits us to use the measured cross-sectional area as an approximation in the above formula. Thickness cancels out, as can be seen in the following complete formulation:

$$\text{DNA concentration in mg} = \frac{E}{\text{thickness in cm}} \times \frac{1}{75{,}000} \times 310 \times 10 \times 10^3$$

$$\text{DNA content in mg} = \text{DNA concentration} \times \text{volume in L.} = \frac{E}{\text{thickness}} \times$$

$$\times \frac{1}{75{,}000} \times 310 \times 10 \times 10^3 \times \text{area in sq. cm} \times \text{thickness in cm} \times 10^{-3} =$$

$$= E \times \frac{31}{750} \times \text{area in sq. cm.}$$



The absorption curve of a typical thymus nucleus stained with methyl green is presented in the figure for comparison with the absorption curves of solutions of methyl green and of the methyl green-DNA complex (from (4)).

DISCUSSION

It will be noted from the table that calf thymocytes were found, on the basis of methyl green staining, to contain between 0.6 and 0.8×10^{-8} mg DNA per nucleus. This value is in agreement with that found by Mirsky and Ris (8) by chemical analysis and counting of isolated nuclei, and by Kurnick & Thorell (7) by densitometry of photographic plates of homogeneous thymus nuclei photographed at several U.V. wave lengths. If the value for the molar extinction coefficient of 100,000 found in *in vitro* experiments for DNA-methyl green (4) had been used, the values would have been 25 per cent lower. The author has elected to accept the chemical and U.V. analyses as a standard and has therefore chosen the extinction coefficient of 75,000 found for methyl green uncombined with DNA. However, it must be pointed

out that the absorption maximum in nuclei is shifted to 645 from 630 mμ as it is *in vitro*. This can be noted from the curves and is also apparent to the eye since the DNA-methyl green complex is blue-green as compared to the yellower green of the uncombined dye.

The curves also present a possible explanation of why the extinction coefficient for the stained nuclei at 645 mμ lies at about 75,000 instead of the 100,000 found *in vitro*. In the *in vitro* experiments, the maximum change in the extinction coefficient, as can be seen in the figure, is attained when the ratio of dye : P is 1:20 (4) rather than the stoichiometric 1:10. The curve for a DNA-methyl green mixture corresponding to 1 dye: 10 P in solution (see figure) is flatter, more closely resembling that of the stained thymocyte and here the molar extinction coefficient is 86,700. Another possibility to be considered is that some of the DNA may be bound to proteins of the nonhistone type, which are not removed by the HCl treatment, and thus fails to combine with methyl green. If such should be the case, it might also be that the proportion of DNA thus bound may vary in different materials, suggesting caution in the general application of the method herein described. Further studies with a variety of materials are in progress. However, it may be said that the acid groups of DNA in the material thus far encountered are almost exclusively bound to histone-type protein.

The pre-treatment with HCl to remove histone is necessary, as can be seen from the much lower values (about one-half) obtained if this step is omitted. Staining of isolated nuclei in bulk (4) also indicated this change by a factor of 2 after HCl treatment.

Some preliminary results with mouse liver are reported. The non-specific absorption, was found to be about 25 per cent (.03–.04) of the total absorption at 645 mμ. Pollister and Leuchtenberger (9), in their independent photometric investigation of methyl green staining of mouse liver nuclei, found lower non-specific absorptions (0.010 to 0.030) and much higher absorption at 625 mμ (0.265 to 0.296) in 5 μ sections. Using their values as though they were the E at 645 mμ (which would in fact, be about 25 *per cent higher*), and assuming a nuclear diameter of 10 μ, this would indicate a DNA content of 1.2×10^{-8} mg. Their absorption measurements for whole nuclei were 0.34 stained and 0.03 unstained, corresponding to 1.0×10^{-8} mg DNA per cell. These results are already somewhat higher than the value of 0.8×10^{-8} mg which we found for mouse liver nuclei by chemical analyses (6). It should be pointed out that Pollister and Leuchtenberger used their stain at pH 6.3 and decolorized in alcohol. Under both of these conditions, we have found methyl green to be very unstable. Their tissues were fixed

in acetic-alcohol, which results in heterogeneous nuclei with well defined chromatin and empty spaces. In our experience, such material is unreliable for photometric analysis. We regard their staining temperature of 56°C not without risk, since 60°C has been found to depolymerize DNA in solution and in nuclei (4). Our experience also emphasizes the importance of carefully controlling temperature during the embedding of tissues. In the experiment included in the table, in which the embedding oven was maintained at a temperature above 60°C, very little staining was obtained, presumably due to the depolymerization of the DNA by heat.

Chicken erythrocytes were found to contain $0.16–0.18 \times 10^{-8}$ mg DNA. This value is somewhat lower than the 0.23×10^{-8} mg found by Mirsky and Ris (8) for fowl liver.

It appears that with proper precautions, methyl green can be utilized for the quantitative estimation of polymerized DNA in nuclei. It should also be of value, therefore in determining changes in DNA polymerization during physiological and pathological processes and as a result of the action of chemical and physical agents.

SUMMARY

1. A method for the quantitative estimation of DNA in cells is described.
2. This method depends upon the preparation of homogeneous nuclei, removal of competing proteins, staining and differentiating at controlled pH. Care in fixation and embedding are important to prevent depolymerization of the DNA which destroys methyl green staining.
3. Several tissues were analyzed in this manner and the results presented.

REFERENCES

1. Caspersson, T., *Symposia of the Soc. for Exp. Biol.*, I. *Nucleic Acid*, **1**, 127 (1949).
2. Hogeboom, G., Schneider, W., & Pallade, G., *J. Biol. Chem.*, **172**, 619 (1948).
3. Kurnick, N. B., *Cold Spring Harbor Symposia on Quantitative Biology*, **12**, 141 (1947).
4. —— *J. Gen. Physiol.*, in press.
5. Kurnick, N. B., & Mirsky, A. E., *J. Gen. Physiol.*, in press.
6. Kurnick, N. B., & Mirsky, A. E., unpublished experiments.
7. Kurnick, N. B., & Thorell, B., unpublished experiments.
8. Mirsky, A. E., and Ris, H., *Nature*, **163**, 666 (1949).
9. Pollister, A. W., & Leuchtenberger, C., *Proc. Nat. Acad. Sci.*, **35**; 111 (1949).
10. Stoneburg, C. A., *J. Biol. Chem.*, **129**, 189 (1939).

ADDENDUM

March 14, 1950

Recent *in vitro* studies (to be published) reveal that whereas the dye used in these experiments (Methyl (Ethyl) Green, ethylated hexamethyl pararosaniline, C. I. No. 685, Certification No. NG 26, National Aniline Corporation) is identical with the methyl green used in our earlier experiments on stoichiometry (Methyl Green, heptamethyl pararosaniline, C. I. No. 684, Certification No. NG 3, National Aniline Corporation) with respect to its absorption spectrum and molar extinction coefficient both in the free state and combined with polymerized desoxyribonucleic acid, the stoichiometric ratio is 13 P per dye molecule as compared with 10 previously found for the dye of Color Index No. 684. Thus, the formula for calculating DNA content should read:

$$\text{DNA} = \text{E} \times \text{Area} \times \frac{31 \times 13}{10{,}000}$$

This obviously yields the same results as those calculated from the formula given in this paper and dispenses with the necessity of using an arbitrary molar extinction coefficient of 75,000 instead of the correct value of 100,000 for the DNA-methyl green complex. The empirical formula thus becomes the same as the theoretical and there is no need to assume differences between the behaviour of the dye in nuclear staining and *in vitro* staining of DNA.

ON THE OXYGEN UPTAKE OF PARACENTROTUS LIVIDUS EGG HOMOGENATES AFTER THE ADDITION OF CALCIUM

TORE HULTIN

The Wenner-Gren Institute for Experimental Biology, Stockholm

Received August 1, 1949

It was shown in a previous paper (6) that in Warburg experiments a sudden oxygen uptake occurs if a small quantity of $CaCl_2$ is added to *Psammechinus* egg homogenates made from calcium-free egg suspensions. This oxygen uptake is considerably more rapid than the uptake produced by addition of hexose monophosphate, malic, fumaric, or succinic acids. It is not influenced by addition of methylene blue or pyocyanine. After about 20 minutes, the whole phenomenon is over, and a lower rate of respiration is gradually obtained in the flasks containing calcium than in the control. A similar effect is obtained by hypertonicity.

CALCIUM AND HYPERTONICITY EFFECTS IN PARACENTROTUS HOMOGENATES

In all experiments the *Paracentrotus lividus* (LM) eggs were freed from jelly by acid treatment and then carefully washed 8 times in Ca-Mg-free artificial sea water (9) containing citrate by means of a hand centrifuge. For each Warburg flask, 1 ml of centrifuged egg mass was prepared and 0.25 ml of 0.35 M sodium citrate solution generally added. The mixture was homogenized by repeated sucking and blowing through a very narrow pipette. The moment of homogenization was noted. For each flask, 1 ml of calcium-free, isotonic Białaszewicz solution[1] (recalculated from Örström (20) was added to the homogenate.

Of this mixture, 2.0 ml were added to each Warburg flask together with 0.2 ml of isotonic phosphate buffer (20) pH 7.3[2] and 0.1 ml of diphosphopyridine nucleotide[3] solution (1 mg/ml). The manometers were closed and the first readings made 15 minutes after homogenization. The temperature of the thermostate was 18° C.

[1] The same effects were obtained with Ca-free or Ca-Mg-free Białaszewicz solutions and with isotonic KCl or NaCl solutions. All solutions were isotonic with the sea water of the Gulf of Naples (cf. 9).

[2] Between pH 6.5 and 8.0, the methylene blue decolorisation rate of *Paracentrotus* egg homogenates increased with the pH.

[3] Kindly supplied by Dr. O. Lindberg.

Fig. 1. Disappearance of the calcium sensitivity in aging homogenates. Addition of $CaCl_2$ (0.003 M) at different times indicated by arrows. (0 minutes = 15 minutes after homogenization.)

The effects of calcium and hypertonicity on homogenates of unfertilized[1] *Paracentrotus* eggs were quite similar to the effects, found earlier in *Psammechinus* (6). Also in *Paracentrotus,* full calcium effect was readily obtained with a total concentration of added calcium of about 0.003 M even in the presence of a tenfold excess of sodium citrate. Full hypertonicity effect was obtained by the addition of 0.25 ml of 25 per cent NaCl to a final volume of 3 ml. The time curves of the oxygen uptake were almost identical for calcium and hypertonicity. Sometimes the oxygen uptake after hypertonicity was slightly higher. Magnesium ions had no influence.

1. Centrifugation Experiments. The centrifugation experiments (6) were repeated with *Paracentrotus* homogenates. The suspensions were more

[1] In homogenates of fertilized (20 minutes) *Paracentrotus* eggs also it was possible to get a calcium effect (Thunberg experiments). No quantitative comparison between the effects in unfertilized and fertilized egg homogenates were performed because of the difficulty in homogenizing fertilized eggs in an incontestable way.

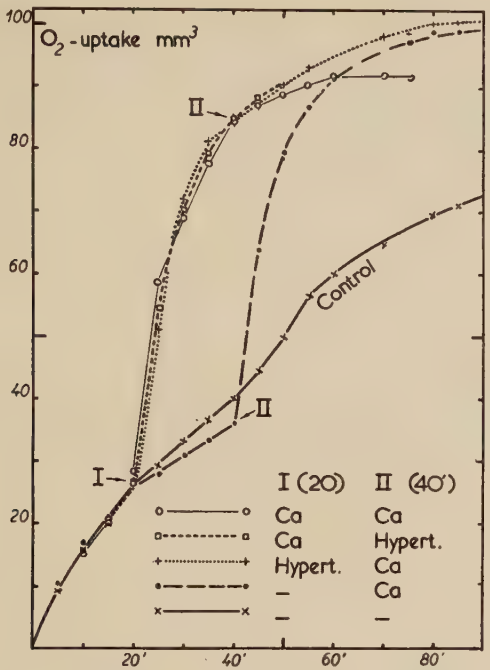


Fig. 2. Repeated addition of $CaCl_2$ and hypertonicity and superimposition of $CaCl_2$ and hypertonicity. ($CaCl_2$ = 0.004 M. Hypertonicity = 0.4 M NaCl excess.)

diluted than previously in the *Psammechinus* experiments (dilution with 15 volumes of Białaszewicz fluid) and the sedimentation was therefore more effective. In Thunberg experiments, the decolorisation rate of the combined sediment and supernatant fluid was considerably higher than that of the isolated supernatant fluid (cf. 8). In *Paracentrotus* also, however, the calcium effect was approximately the same in both cases and thus not localised to the large cell granules.

2. *Duration of the Calcium and Hypertonicity Sensitivity.* Oxygen uptake as a result of calcium addition or hypertonicity could be obtained only in quite fresh homogenates. In homogenates prepared as described above, full effects were generally obtained within 45–60 minutes after homogenization. Between 1–1 ½ hours after homogenization (18° C), the effects rapidly diminished and disappeared (Figure 1).[1]

[1] Just before the end of the calcium-sensitive period in the *Paracentrotus* homogenate, a spontaneous increase in respiration was very often observed (Figures 1, 2) in the calcium-free control. In *Psammechinus* this phenomenon was not seen.

3. Superposition of Calcium and Hypertonicity. If calcium was added to homogenates and a new calcium addition was made just after the end of the first oxygen uptake, no second uptake was produced. Nor was it possible to repeat the hypertonicity oxygen uptake. The calcium and hypertonicity effects have fundamental mechanisms in common, for they could not be superimposed on one another (Figure 2).

ON THE MECHANISM OF THE OXYGEN UPTAKE

1. Glycogen Analysis.

Quite fresh homogenate was divided into two parts. $CaCl_2$ solution was added to one of them (final concentration 0.005 M). Samples were taken immediately before Ca-addition and after ½, 1, 2, 3, 5, 7, and 10 minutes and transferred directly into equal volumes of 60 per cent KOH. Glycogen was determined as described by Lindberg (10).

During this short initial period of calcium-produced oxygen uptake, the glycogen[1] breakdown was very small and no difference could be found between calcium-free and calcium-treated homogenates.

2. Respiration Quotients of the Calcium and Hypertonicity Effects.

The respiration quotients were determined according to the "phosphate method" (2). $CaCl_2$ (0.003 M) was added after 20 minutes and HCl in excess after 30 minutes.

In fresh, calcium-free *Paracentrotus* homogenates there was a strong formation of carbon dioxide exceeding the oxygen uptake by 50–75 per cent (Tables 1, 2). Within the limits of error, the oxygen uptake after calcium addition or hypertonicity treatment was not accompanied by any increase in the carbon dioxide output.[2] In several experiments, an inhibition of the carbon dioxide formation was found after the addition of calcium.

3. Phosphate Metabolism.

Paracentrotus egg homogenate prepared as described above was divided into two parts. Isotonic (0.398 M) $CaCl_2$ solution was added to one part to a final concentra-

[1] The "glycogen" fraction in the sea urchin egg probably includes mucous substances (15, 19).

[2] In the first paper on the calcium-effect (6), model experiments with trypsin were described in which it was shown that when trypsin is added to quite fresh *Psammechinus* homogenates, the respiration of the homogenates is stabilized at a higher level. This has also been shown to be the case in *Paracentrotus* homogenates (Crystalline trypsin and chymotrypsin (Armour Lab)). The RQ of the trypsin respiration was the same as that of normal homogenates.

Papain (Lemke & Co.) on the other hand gave a lower respiration rate. It was possible to get a normal calcium produced oxygen uptake in homogenates treated with these proteolytic enzymes.

Table I

Oxygen Uptake and Carbon Dioxide Formation after the Addition of Calcium.[1]

19/2 1949	Ca-free Homogenate	$CaCl_2$ (0.003 M) added after 20 minutes
CO_2 µl.	53.9	55.0
O_2 »	33.3	61.6
RQ.....	1.62	0.89
RQ of Ca-effect: $\frac{1.1}{28.3}$ = 0.039		

Table II

Oxygen Uptake and Carbon Dioxide Formation in Hypertonicity.[1]

23/3 1949	Isotonic Homogenate	Hypertonicity (0.35 M excess NaCl) after 20 minutes
CO_2 µl.	50.2	51.8
O_2 »	28.8	65.8
RQ.....	1.75	0.79
RQ of hypertonicity effect: $\frac{1.6}{37.0}$ = 0.043		

tion of 0.005 M. Samples were taken immediately before Ca-addition and after ½, 2, 5, and 10 minutes and transferred into 10 per cent trichloroacetic acid (TCA). After repeated washing, the combined supernatants[2] were analysed for orthophosphate, labile phosphate and total phosphate (11).

During the short time of the experiment, the orthophosphate fraction increased about 5 per cent (mean value). No significant difference was obtained in the acid-soluble phosphate fractions between the calcium-free and calcium-treated homogenates.

It was shown by Lindberg & Ernster (13) that sea urchin egg homogenates can utilise oxidation of glucose for esterification of phosphate. The oxygen uptake after the addition of calcium cannot be used for phosphate esterification, however. In experiments with phosphate labelled with P^{32} it was found that the phosphorylation was retarded after calcium addition[3] (Table 3).

[1] For technical reasons only half homogenate concentrations were used in the RQ experiments.

[2] For unknown reasons, the TCA-extracts from Ca-homogenates were slightly opalescent, even after prolonged centrifugation.

[3] This was also the case in trypsin-containing homogenates.

Table III

P^{32} contents of the Acid Soluble Phosphate Fractions 5 Minutes after the Addition of Calcium. (Impulses/minute).

	Ca-free Homogenate	Ca-homogenate
Orthophosphate fraction	647	677
Orthophosphate + labile phosphate	727	741
Labile phosphate fraction isolated	76	60
Total phosphate	734	730

Phosphate[1] containing P^{32} was added to calcium-free homogenate. The homogenate was divided into two parts. Five minutes after the isotope addition, $CaCl_2$ was added to one part to a concentration of 0.005 M. Samples were taken 5 and 15 minutes after Ca-addition and transferred into 10 per cent TCA. After washing the combined supernatants were collected and fractionated for activity determinations as described by Lindberg (11). The activities of the isolated labile phosphate fractions were determined according to Lindberg (12).

4. Inhibition of the Oxygen Uptake by Iodoacetate.

The respiration of *Strongylocentrotus dröbachiensis* egg homogenate is not inhibited but rather slightly stimulated (13) by the addition of monoiodoacetate (0.005 M). In calcium-free *Paracentrotus* homogenate monoiodoacetate exerted a weak, progressive inhibition of the respiration. This was true even in the presence of methylene blue or pyocyanine.

The strong inhibitory effect of iodoacetate on the calcium- or hypertonicity-produced oxygen uptake was much more striking, however. In the presence of 0.005 M iodoacetate the inhibition was evident already after 5 minutes and in most cases complete after about 30 minutes (Figure 3).

5. Demonstration of Unmasked Sulfhydryl Groups by Nitroprusside Test.

The reactions occurring in *Paracentrotus* egg homogenates under the influence of calcium are very delicate and difficult to investigate directly by chemical analyses because of their complexity and the short duration of the calcium sensitive period. The following nitroprusside experiments indicate changes in soluble sulfhydryl groups after the addition of calcium.

Paracentrotus homogenates diluted 1 : 10 by isotonic KCl solutions were centrifuged for 20 minutes at 2,000 g in a cold room. From the weakly opalescent supernatant fluid two samples of 5 ml were taken. $CaCl_2$ (0.005 M) was added to one of them. After about ½ minute 0.1 ml of 5 per cent sodium nitroprusside and 0.2 ml

[1] Kindly supplied by Dr. O. Lindberg.

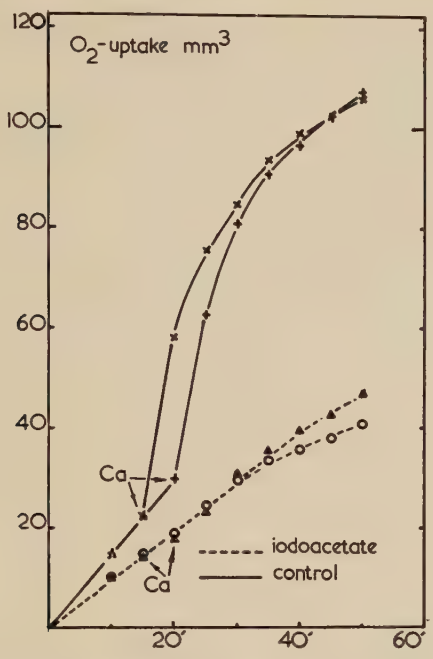


Fig. 3. Influence of iodoacetate (0.005 M) on the oxygen uptake in *Paracentrotus* egg homogenates after the addition of calcium. Iodoacetate added immediately after homogenization. Incubation times 30 and 35 minutes (0 minutes = 15 minutes after homogenization). Addition of $CaCl_2$ (0.003 M) indicated by arrows.

of M NH_4OH were added to both samples. The weak color was read directly in a Klett & Summerson (18) colorimeter (filter KS 54).

In the tubes containing calcium a slightly higher extinction was constantly obtained. The mean value from a number of experiments corresponds to an uncovering of sulfhydryl groups approximately equivalent to 0.02 mg of cystein. The individual values were, however, very variable.

DISCUSSION

Carbohydrates have been found to be the most important substrates for the oxydative metabolism of sea urchin egg homogenates. In homogenates glycogen is rapidly broken down (21). Glucose (13), hexose-6-phosphate, phosphogluconic, and malic acids (10) can be used as substrates for respiration.

However, the evidence presented in this paper shows that the transient and very rapid oxygen uptake immediately after the addition of calcium or

hypertonicity to *Paracentrotus* egg homogenates cannot be due merely to an acceleration of the oxidative carbohydrate metabolism. This oxygen uptake is not accompanied by any carbon dioxide production, which would be the case if carbohydrates were metabolized. Neither can the oxidation energy be utilised for the esterification of phosphate. No accelerated glycogen breakdown could be found.

A sudden oxidation of hydrogen carriers (especially diphosphopyridine nucleotide which is present in the sea urchin eggs in sufficient amounts (17, 3) might conceivably be an explanation. Diphosphopyridine nucleotide is, however, oxidized quantitatively in the presence of pyocyanine (7), while the calcium and hypertonicity effects are quite insensitive to the presence of pyocyanine. Addition of diphosphopyridine nucleotide to the homogenates, which was made in most experiments, had no influence on the effects.

The sensitivity of the calcium and hypertonicity effects to monoiodoacetate is taken as an indication that the oxygen uptake described is due to or transmitted by substances containing sulfhydryl groups. In *Paracentrotus* and *Psammechinus* homogenates the calcium effects are accompanied, especially anaerobically, by a mercaptane-like odour. To a lesser degree, this is true after hypertonicity treatment also. The phenomenon does not occur in the presence of iodoacetate or $CuSO_4$ (0.001 M).[1] Most likely, sulfhydryl groups are successively unmasked and oxidized as a result of the addition of calcium. The nitroprusside experiments lend support to this assumption. It has already been shown in a previous paper (6) that the calcium effect is accompanied by a strong increase in the viscosity of the homogenates. A following study will demonstrate that the viscosity is increased under the influence of $CuSO_4$ or iodoacetate also, most likely by means of a transformation of protein-bound —SH to S—S.[2] The suggestion has been made by Heilbrunn (4) that calcium in the sea urchin eggs is bound to a large extent to proteins. These facts, as well as the high ionic specificity of the reaction, lend some support to the hypothesis that sulfhydryl groups unmasked by calcium in *Paracentrotus* and *Psammechinus* egg homogenates[3] are supplied by proteins[4].

The relation between free and bound calcium in the sea urchin eggs has been investigated by several authors (14, 16, 22). These experiments indicate that most of the calcium in unfertilized eggs is bound to non-dialysable substances. The present investigation shows the very strong affinity of the

[1] In centrifuged homogenates the odour was produced only in the supernatant fluid.
[2] Cf.: $2\ RSH + 2\ CH_2ICOOH \rightarrow RS\text{-}SR + 2\ HI + C_2H_4(COOH)_2$ (1).
[3] *Arbacia lixula* (L.) egg homogenates behave in a different way.
[4] Cf. Hopkins (5).

Paracentrotus egg homogenates to calcium. Full calcium effect is released by about 0.003 M $CaCl_2$ in a tenfold excess of sodium citrate. Since the calcium-binding capacity of the homogenate is so great, it seems very probable that even in intact, unfertilized eggs free calcium can occur only in very small concentrations.

The calcium sensitivity of *Paracentrotus* egg homogenates disappears in aging homogenates. The form of the respiration curves (Figures 1, 2) of calcium-free homogenates indicates that this disappearence might be related to oxidative processes. Such processes are, however, not the only cause. Homogenates kept anaerobically for a certain period also lose their sensitivity to calcium though in most cases slightly later than aerobic homogenates.

The unfavourable influence of calcium ions on the metabolism of egg homogenates makes it important to use egg homogenates made from calcium-free egg suspensions in metabolic studies.

SUMMARY

1. After the addition of small amounts of calcium to *Paracentrotus* egg homogenates made from Ca-free egg-suspensions there is a sudden, very rapid oxygen uptake lasting about 20 minutes. The same phenomenon has previously been found in *Psammechinus*.

2. Hypertonicity is so far the only model procedure for obtaining this same effect. The calcium and hypertonicity oxygen uptakes behave alike in all respects. They cannot be superimposed on one another. Evidently they are released over the same mechanisms.

3. The sensitivity of the *Paracentrotus* egg homogenates to both calcium and hypertonicity disappears between 1–1 ½ hours after homogenization (18° C).

4. The oxygen uptake investigated is not caused by an increased glycogen breakdown. Carbohydrate metabolites are not involved, for the oxygen uptake is not linked to any carbon dioxide production. The oxidation energy cannot be used for esterification of phosphate. Within the limits of error no difference in the acid soluble phosphate fractions could be found during the initial 10 minutes of the calcium-produced oxygen uptake between calcium-free and calcium-treated homogenates.

5. The oxygen uptake is not caused by an oxidation of diphosphopyridine nucleotide, for it is equally evident in the presence of pyocyanine.

6. Monoiodoacetate inhibits the oxygen uptake. This fact is taken as evidence that the effect is due to or transmitted by substances containing sulfhydryl groups. By nitroprusside test a certain unmasking of sulfhydryl groups was indicated after the addition of calcium to homogenates. Some evidences have been collected in support of the hypothesis that under the direct or indirect influence of calcium protein-bound sulfhydryl groups are uncovered and oxidized.

7. The great affinity of egg homogenates to calcium is shown by the fact that full calcium effect is produced by about 0.003 M $CaCl_2$ even in a tenfold excess of sodium citrate.

8. Because of the inhibiting effect of calcium on the metabolism of egg homogenates, metabolic studies should be carried out on homogenates prepared from calcium-free egg suspensions.

Acknowledgements: The investigation was carried out at the Zoological Station of Naples. The author wishes to express his deep gratitude to the Station Staff for laboratory facilities and materials. He wishes to express his most cordial thanks to Professor J. Runnström for all help and keen interest, and to Fil. Mag. Karin Hultin for valuable assistance. The expences of this investigation were defrayed by grants awarded to the Wenner-Gren Institute by the »Swedish Natural Science Research Council».

REFERENCES

1. Bull, H. B., *Adv. Enzymology*, **1**, 1 (1941).
2. Dickens, F., & Šimer, F., *Biochem. J.*, **24**, 905 (1930).
3. Goldinger, J. M., & Barron, E. S. G., *J. Gen. Physiol.*, **30**, 73 (1946).
4. Heilbrunn, L. V., *An Outline of General Physiology*, London, 1937.
5. Hopkins, F. G., *Biochem. J.*, **19**, 787 (1925).
6. Hultin, T., *Arkiv Kemi, Mineral., Geol.*, **26** *A*, No. 27 (1949).
7. Hummel, J. P., Unpublished.
8. Hutchens, J. O., Kopac, M. J., & Krahl, M. E., *J. Cellular Comp. Physiol.*, **20**, 113 (1942).
9. Hörstadius, S., *Pubbl. Staz. Zool.* Napoli, **14**, 251 (1935).
10. Lindberg, O., *Arkiv Kemi, Mineral., Geol.*, **16** *A*, No. 15 (1943).
11. —— *Arkiv Kemi, Mineral., Geol.*, **23** *A*, No. 2 (1946).
12. —— *Arkiv Kemi, Mineral., Geol.*, **26** *B*, No. 13 (1948).
13. Lindberg, O., & Ernster, L., *Biochim. Biophys. Acta*, **2**, 471 (1948).
14. Mazia, D., *J. Cellular Comp. Physiol.*, **10**, 291 (1937).
15. Monné, L., *Arkiv Zoologi*, **1**, No. 6 (1949).
16. Monroy-Oddo, A., *Experientia*, **2**, 1 (1946).
17. Runnström, J., *Biochem. Z.*, **258**, 257 (1933).
18. Summerson, W. H., *J. Biol. Chem.*, **130**, 149 (1939).
19. Vasseur, E., *Acta Chem. Scand.*, **2**, 900 (1948).
20. Örström, Å., Z. *Physiol. Chemie*, **24**, 1 (1941).
21. Örström, Å., & Lindberg, O., *Enzymologia*, **8**, 367 (1940).
22. Örström, Å., & Örström, M., *Protoplasma*, **36**, 475 (1942).

THE STRUCTURE OF THE NUCLEUS OF SACCHAROMYCES BAYANUS

CARL C. LINDEGREN and MARGARET M. RAFALKO

Southern Illinois University, Carbondale, Illinois

Received August 21, 1949

INTRODUCTION

The salient features of the fungal nucleus were clearly established by Harper (13) in his classical work on *Phyllactinia*. He showed that it is a highly polarized structure with a densely chromatic centrosome located at the periphery of a large nuclear vacuole with the chromosomes attached to the centrosome. The nucleolus was also a conspicuous structure in his description as a "sharply defined oval body" within the nucleus; the "antipolar ends" of the chromosomes were often in contact with the nucleolus. This observation is consistent with the modern view that the nucleolus originates from a specific chromosomal region.

Wager and Peniston's (42) description of the yeast nucleus corresponds in many respects to Harper's description of the nucleus of *Phyllactinia*. They described a large nuclear vacuole containing a "central volutin granule", which is the true nucleolus (J. S. Rafalko, unpublished), with an attached eccentric structure which they called the "nucleolus" to which the "chromatinic threads" were polarized as is characteristic of most fungal nuclei. Janssens and Leblanc (18) previous to Wager and Peniston had characterized the large vacuole as the nucleus and had correctly designated the "crystalloid" of the vacuole as the nucleolus. In their study of the nucleus of *Neurospora*, Dodge (6) and Lindegren and Ruman (26) discovered that it also was a nucleus characterized by a conspicuous eccentric structure Dodge called the beak to which the chromosomes were polarized. Lindegren and Ruman showed that the nucleolus was attached near the end of one chromosome in the nuclear vacuole of *Neurospora*. This was subsequently confirmed by McClintock (29).

Guilliermond (10) in a polemic with Wager ridiculed the significance

of his designations and insisted that the deeply staining eccentric structure attached to the nuclear vacuole was actually the nucleus. Even cytologists who duplicated the results of Wager and Peniston as is evidenced by their figures, for example Kater (14) and Henneberg (19), have followed Guilliermond in their interpretations, probably due to the great prestige he held.

The reason for the general agreement with Guilliermond is probably that the structure called the nucleolus by Wager and Peniston, the nucleus by Guilliermond and the centrosome by Lindegren is easy to demonstrate with any cytological method while the chromatinic threads of Wager and Peniston or chromosomes of Lindegren are more difficult to stain. Only the centrosome gives a positive reaction with the standard Feulgen method. However, a modification of the Feulgen technique was devised by Rafalko (32), who demonstrated that the "chromatinic threads" of Wager and Peniston are Feulgen-positive and identified them as chromosomes.

MATERIALS AND METHODS

The yeast cultures were grown at room temperature on agar slants. When slides were to be made, a flask of liquid medium was inoculated with yeast from a 24-hour slant and incubated at 30° C for one to six hours on a shaker. After centrifuging a sample from the flask, cover-slip smears were made without albumen and were fixed by putting them into a Petri dish of fixative. Throughout the staining procedure the cover-slips were kept immersed in some fluid until mounted on a slide. At no time was the material allowed to dry.

The fixatives most commonly used were Navashin, Flemming, Carnoy, Bouin, mercuric chloride, and formalin and osmic acid vapors. Best results were obtained with Navashin and Carnoy. Staining procedures employed included the Feulgen reaction, methyl green — acid fuchsin, methyl green — pyronin y, safranin O-fast green, crystal violet-anilin blue, Giemsa, Sudan IV, and Heidenhain's haematoxylin. With the exception of the Heidenhain stain, Sudan IV, and the sulphurous acid and Schiff reagent of the Feulgen reaction, all fixatives, stains, and dehydrating reagents were made up immediately prior to use. Below is a summary of the stain reactions. Note the similarity of the picture presented after methyl-green-pyronin y to that after the Feulgen reaction. This seems especially significant since methyl green is recognized as a good chromatin stain (3, 31) however, we find that

in the yeast cell methyl green can be washed out unless differentiation and dehydration are accomplished rapidly.

TABLE I

Stain	Feul–FG	MG–AF	MG–Py	Saf O–FG	Giemsa	CV–AB after KI mordant	Sudan IV	Heid. Haem.
Centrosome	Feul. + early in growth cycle[1]	Red	Pale green	Red	—	Red-blue	—	Gray-black
Chromosomes	Feul. + later in growth cycle[1]	Dark green	Dark green	Red	Purple-red	Deep Purple	At times	Deep blue-black
Nucleolus	Pale green	Pale green	Pale green	Green	Pale blue	Pale blue	At times	Gray
Cytoplasm	Pale green	Red	Red	Green	Pale blue	Pale blue	Cyto-plasmic bodies	Pale gray, almost colorless

OBSERVATIONS

Description of the Organism

S. bayanus is an elongate cell whose cytoplasm appears homogeneous in the living state. The nucleus or nuclear vacuole is of the same shape as the cell itself. Within this nuclear vacuole are four or five pairs of chromosomes and the nucleolus, a large homogeneous body occupying the center of the nuclear vacuole. The nucleolus is most often of the same general shape as the nuclear vacuole, though in very young cells it sometimes appears round. The contracted chromosomes are small rod-shaped bodies. At one side of the vacuole lies the large chromatic centrosome whose position allows its close relationship with the chromosomes and, at the same time, with the cytoplasm.

Fixed and Stained Material

The observations discussed here are all based on methyl green-acid fuchsin preparations and the Feulgen reaction, for which the modified method of Rafalko (32) was used exclusively.

[1] Only the Feulgen reaction shows this cyclic relationship in centrosome-chromosome staining.

NUCLEAR VACUOLE

A. *Chromosomes.* The chromosomes number four or five pairs and stain dark green with methyl green. Either the chromosomes (Fig. 9) or the centrosome (Figs. 1–8) stain Feulgen-positive, for only rarely are cells found in which both the centrosome and the chromosomes are Feulgen-positive. The Feulgen-positive substance of the chromosomes is red-red and sometimes somewhat refractile. Chromosomes are often visibly attached to the nucleolus (J. S. Rafalko, unpublished). The chromosomes may be distributed throughout the vacuole (Figs. 9 and 16), in close association with the centrosome (Fig. 10) or some near the centrosome and some near the *cytoplasmic clear area,* p. 147, (Figs. 11, 13 and 15).

B. *Nucleolus.* The nucleolus generally takes on the cytoplasmic stain (Fig. 1) with the exception of the procedures involving the use of methyl green. On rare occasions it appears pink following Feulgen. With methyl green, in combination with acid fuchsin or pyronin y, the nucleolus stains a pale green, the chromosomes dark green, the cytoplasm red. The intensity of the dye in the nucleolus is too low, however, to be significant. A large nucleolus always appears early in the growth cycle (Figs. 1 and 12). The shape of the nucleolus varies with the shape of the nuclear vacuole or the cell, and it generally has a central location in the nuclear vacuole. Chromosomes when visible are often attached to the nucleolus. At those times in the growth cycle when the large nucleolus characteristic of growing cells is visible, chromosomes are not often seen. When the chromosomes stain clearly, a very small nucleolus is often found in close association with one pair of chromosomes (Fig. 11 and bud of Fig. 16).

C. *Nuclear Membrane.* The presence of a definite nuclear membrane is indicated by the regular outline of the nuclear vacuole rather than by actual observation of a definite structure. In preparations stained with acid fuchsin (Figs. 10–16) a definite membrane encloses the centrosomal area and appears to be continuous with the nuclear membrane. After Carnoy fixation this continuity is not obvious (Figs. 1, 4–6), though with this fixative the nuclear vacuole and its contents usually are not well preserved. After Navashin fixation, which was found to be especially good for the nuclear vacuole and its contents, the centrosome appears to be within the nuclear membrane (Figs. 9–11, 13, 14, 16). However, some variation occurs after this fixative. In Fig. 10 the fact that the chromosomes lie just at the periphery of the faint-staining halo around the centrosome proper indicates that the material of the area comprising the halo is more gelatinous than the

nuclear sap and may form a pocket in which the centrosome lies. Figs. 9, 12, and 13 show a constriction of the vacuolar membrane at the centrosomal area, from which it seems to continue on around the centrosome. The mother cell of Fig. 15 shows a sharper delineation of centrosomal area and nuclear vacuole proper.

CENTROSOME

The present authors have identified the centrosome as the highly chromatic spherical body located at one side of the nuclear vacuole (Figs. 1 and 4). The centrosome persists throughout both resting and division stages and is thus a permanent structure throughout the life history of the vegetative cell.

In the preparations stained with acid fuchsin the appearance of the centrosome is invariable as a homogenous, red, spherical body containing a *central clear sphere* (Figs. 10–12, 14–16). It is immediately surrounded by a less densely stained area and the whole structure seems to lie within the boundary of the membrane of the nuclear vacuole (Fig. 10). This same picture is observed in methyl green — pyronin y and in safranin O — fast green preparations. The position of the centrosome in the cell varies. Most frequently it is near the point of budding (Fig. 15), though it may be medial (bud of Fig. 16) or may appear in the cell opposite the bud (Fig. 15). The *central clear sphere* of the centrosome is sometimes double (Fig. 13), and a picture similar to Fig. 6 of the Feulgen preparations is often seen after methyl green-acid fuchsin, with a *clear sphere* in each of the two bodies.

As has been stated, either centrosome or chromosomes may be Feulgen-positive. The Feulgen-positive substance of the centrosome is a purple-red and varies in form and position (Figs. 1–7), thus differing from the invariable appearance obtained with methyl green-acid fuchsin. The internal structure of the centrosome revealed by the Feulgen reaction is brought out very well after Carnoy. Sometimes the entire surface of the centrosome is stained (Fig. 4) while at other times the Feulgen-positive substance is limited to a crescent at one side of the centrosome (Figs. 1 and 2). This chromatin may assume various shapes and often appears to be passing into the bud (Fig. 5). The *central clear sphere* of the centrosome is in close association with the Feulgen-positive substance, especially if the latter is in the form of a crescent (Fig. 1) when the *central clear sphere* is often double. The Feulgen-positive substance often appears to be double (Fig. 6). The separating Feulgen-positive bodies are connected by a strand which stains with the cytoplasmic dye (Fig. 8). This strand was also reported by Janssens and

Leblanc and by Swellengrebel (Figs. 29 and 35). The picture of Fig. 8 somewhat resembles the structure called a desmose[1] by protozoologists.

CYTOPLASMIC INCLUSIONS

In fresh, living cells observed in water mounts, many small granules are visible throughout the cytoplasm and have been identified as mitochondria. Careful attention to focal level reveals that the mitochondria are in the cytoplasm and are most numerous at the outer periphery of the centrosome and the nuclear vacuole. They often are aggregated in two clumps: one near the centrosome and one near region of the *cytoplasmic clear area*. Mitochondria usually are not visible in fixed and stained preparations.

PLATE I →

Figures 1–9. Feulgen-fast green.

Fig. 1. Chromo-aceto-formol fixation. 1½ hrs. growth. Feulgen-positive substance of centrosome is in crescent form and the centrosomal clear sphere is in close association with it. The well preserved vacuole contains a large faint green nucleolus.

Figs. 2–7. Carnoy fixation. 1½ hrs. growth. These figures show variation of pattern of the Feulgen-positive substance of the centrosome. Fig. 4 shows Feulgen-positive bodies similar to Renaud's centrioles. Fig. 7 shows centrosomal mass elongated between two masses of Feulgen-positive substances.

Fig. 8. Chromo-aceto-formol fixation. 1 ½ hrs. growth. The two Feulgen-positive masses of the centrosome have a Feulgen-negative strand between them.

Fig. 9. Chromo-aceto-formol fixation. 6 hrs. growth. Feulgen-positive chromosomes visible in nuclear vacuole. Note centrosome-nuclear vacuole relationship.

Figs. 10–16. Methyl green-acid fuchsin. Chromo-aceto-formol fixation. 1½ hrs. growth.

Fig. 10. Paired chromosomes in close association with centrosomal area. Centrosome with surrounding lightly stained halo seems to be within vacuolar membrane. Central clear sphere in centrosome. Clear area in cytoplasm at end of nuclear vacuole opposite centrosome.

Fig. 11. Same centrosomal-nuclear vacuole relationship apparent. Centrosome shows central clear sphere. Clear area in cytoplasm opposite centrosome. Half of chromosomes near centrosomal area; half near the clear area at opposite end of the nuclear vacuole where they are clustered around a small nucleolus.

Fig. 12. Pale green nucleolus but no chromosomes visible in the nuclear vacuole. In this cell the vacuolar membrane appears slightly constricted in centrosomal area. Centrosomal clear sphere and opposite cytoplasmic clear area visible.

Fig. 13. Centrosomal clear sphere double. Outline of nuclear vacuole vague, but the membrane appears to constrict somewhat at centrosomal area, then continue on around the centrosome.

Fig. 14. Centrosomal areas of mother cell and bud at end of cells away from point of budding. Complete chromosome complement not visible.

Fig. 15. Centrosomes of mother cell and bud adjacent to one another. Centrosome and nuclear vacuole more sharply separated in mother cell. Maximum number of chromosomes visible in mother cell.

Fig. 16. Centrosome of bud mid-way toward cell end opposite the point of budding. Paired chromosomes in both cells. In the bud one pair of chromosomes is in contact with small nucleolus. Mother cell shows clear area in cytoplasm opposite centrosomal area.

[1] According to Kudo a desmose is a connecting strand between dividing centrioles.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16

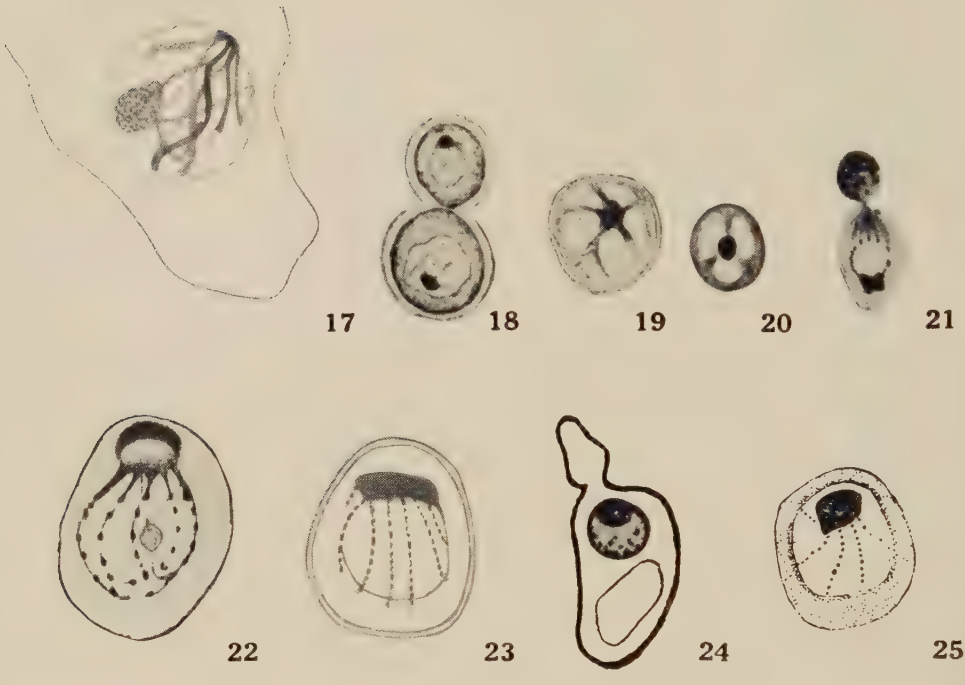


PLATE II

These figures, except where otherwise noted, are photographic copies from the papers of the named authors. The figures were selected because the cells are of similar structure and are presented to show that the same structures have been given different designations by the various authors. The fixative and stain are indicated in those cases where they were definitely designated for the specific figure.

Fig. 17. Harper (13), drawing of his Fig. 49). Flemming's triple stain. Nucleus *Phyllactinia*. The eccentric structure he calls the central body; from it the chromatin strands radiate into the nuclear vacuole which contains a conspicuous nucleolus.

Fig. 18. Janssens and Leblanc (18), Fig. 1 Ie). They designate the vacuole as the nuclear vacuole and the deeply staining structure as the nucleolus.

Fig. 19. Hoffmeister (16), Fig. 2). Von Rath and Heidenhain's haematoxylin. The darkly stained body is called the nucleus. It corresponds to what we designate the centrosome attached to the vacuole.

Fig. 20. Swellengrebel (40), Fig. 3). The dark body is called the resting nucleus. His figure corresponds closely to that presented by Hoffmeister.

Fig. 21. Fuhrmann (7), Fig. 17). He designates this figure as one showing a cell with a well-developed bud into which the daughter nucleus is migrating. He states that two daughter nuclei are present in the mother cell lying close to the vacuole. The strands and particles at the upper edge of the vacuole extending into the bud he regards as remnants of the achromatic spindle.

Fig. 22. Wager and Peniston (42), Fig. 116). Drawn from a preparation fixed in Perenyi's fluid forty-eight hours in Pasteur's solution (foam) to show the volutin granule inside the nucleus.

Cytoplasmic clear area. In the cytoplasm at the end of the nuclear vacuole opposite the centrosome, a *clear area* is visible in nearly every cell (Figs. 10–12 and 16), apparently in contact with the nuclear vacuole. The significance of this observation has not been worked out.

With Sudan IV and Sudan black there is much stainable material in the cytoplasm indicating that much lipoidal material is present in these cells at certain periods during growth.

Living Material

The living cells were observed by two methods a) a drop of dilute yeast suspension was added to a drop of water on a slide, smeared and covered with a cover-slip and b) a very dilute yeast suspension was smeared over a square of melted agar on a slide and this was then covered with a cover-slip. In both methods the cover-slip was ringed with paraffin so that the preparations could be observed over a period of hours and the agar mounts watched during growth.

The structures visible in stained preparations are not demonstrable very regularly in living cells; however, the nuclear vacuole is always visible and the chromosomes often can be seen. The centrosome occasionally is observed as a more opaque area in the cytoplasm while the nucleolus is seldom visible. Mitochondria are readily apparent in the cytoplasm at the outer periphery of the nuclear vacuole, at the centrosomal area and in the region of the *cytoplasmic clear area.*

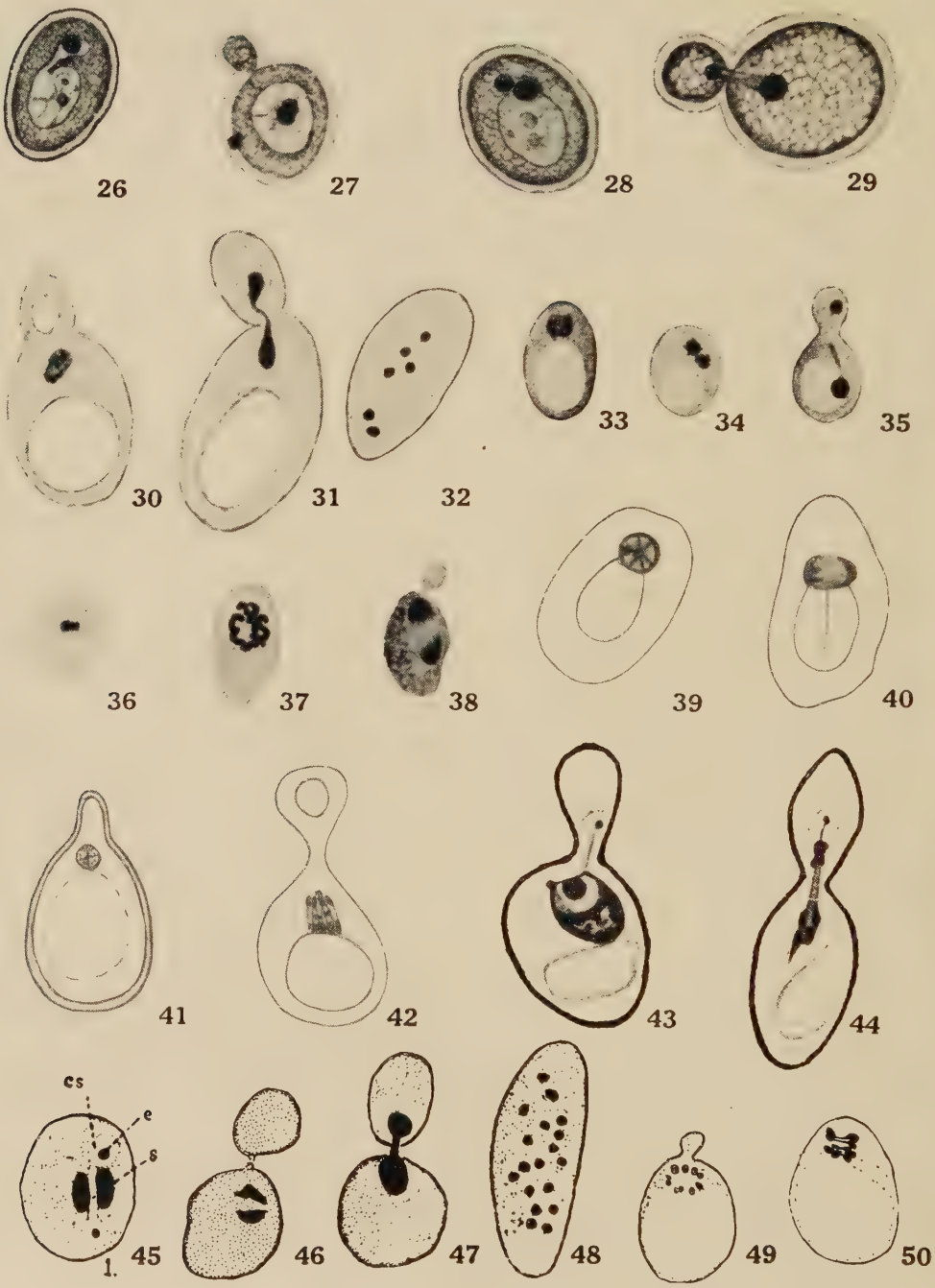
The nucleolus is always visible in killed cells from *growing* cultures. Smears of living cells in water were observed after adding a loop of dye or

---

Fig. 23. Kater (19), Fig. 2). Corrosive sublimate-acetic-alcohol, Heidenhain's haematoxylin. He regards the darkly stained body as chromatinic material in the cytoplasm obscuring the true nucleus. He regards what seem identical with Wager and Peniston's chromatinic threads as granular lines of chromatin material in the cytoplasm. This interpretation is discussed in detail in the text.

Fig. 24. Renaud (33), Fig. 1). Picro-formol, Heidenhain's haematoxylin. The spherical body, called the resting nucleus with a structure identical to that described by Guilliermond, is limited externally by a stained membrane and contains an achromatic nucleoplasm in which is distinguished a nucleolus and some chromatin. The vacuole he refers to as a storage vacuole.

Fig. 25. Subramaniam (37), Fig. 6). Carnoy or Bouin fixation, Heidenhain's haematoxylin. The deeply stained body he calls the central chromatin granule of the nucleus, in this case the large vacuole. The granular lines coming out from the chromatin granule he regards as radiating linear rows of granules.


26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
cs
e
s
l.
45
46
47
48
49
50

←

Plate III

These figures, except where otherwise noted, are photographic copies from the papers of the named authors. They illustrate the different authors' interpretations of cell structures and cell division. The descriptions given are those of the respective authors.

Figs. 26–29. Janssens and Leblanc (18), Fig. 2 II a, 1 I, 2 IV a).
Fig. 26. After 4 hrs. 30 min. in wort. The nucleolus is shown lodged in a fold of the nuclear membrane.
Fig. 27. After 24 hrs. in wort. The central body of the nuclear vacuole they called the nucleolus and stated it was surrounded by a clear or lightly stained halo. They believed the nucleolus to be composed principally of nuclein but also containing a substratum of an albuminoid substance analogous to plastin.
Figs. 28, 29. During budding the nucleolus elongates and divides; the two go toward cell extremities. At this moment they are united by a filamentous structure, a sort of spindle. This description and that of Fig. 26 leads us to believe that Janssens and Leblanc confused nucleolus and centrosome, calling both nucleolus.
Figs. 30–32. Hoffmeister (16), Figs. 5, 6, 9).
Fig. 30. Next to the vacuole lies the stained nucleus, containing a small dark body which he designates as the nucleolus.
Fig. 31. The nucleus, in the region of the bud, has divided into two of which one has migrated into the young bud. The dividing nuclei are bound together by a thin stained band.
Fig. 32. Stage of sporulation of S. ellipsoideus. Compare this figure and description with that of Fig. 48.
Figs. 33–35. Swellengrebel (40), Figs. 18, 20, 22).
Fig. 33. Diaster, the linin is stained.
Fig. 34. Daughter nuclei separating from one another, still united by a linin filament.
Fig. 35. Anaphase.
Figs. 36–38. Fuhrmann (7), Figs. 5, 8, 16).
Fig. 36. This figure shows a cell with a nucleus in which the chromosome number (4) is distinctly detected and appears to be enclosed by achromatic substance.
Fig. 37. A cell in which the split chromosomes are visible.
Fig. 38. A cell with a young bud. In the mother cell the karyokinesis is almost completed. The achromatic spindle is already breaking and its remnants are still attached to the daughter nuclei.
Figs. 39–40. Wager and Peniston (42), drawings of their Figs. 65, 69). These figures show appearances strongly suggestive of nuclear structure and mitotic phenomena. They "... are remarkable for the peculiar distribution of a large amount of deeply stainable substance on the surface of the nucleolus ..."
Figs. 41–42. Kater (19), drawings of his Figs. 14, 20).
Fig. 41. Early bud. Extremely large vacuole.
Fig. 42. Anaphase. Spindle very evident.
Figs. 43–44. Renaud (33), Figs. 3, 12).
Fig. 43. The nucleolus and the chromatin network are situated in very clear regions. — Centrosome divides, one of the resulting daughter centrosomes remains in mother cell — while the second migrates to the bud surrounded by a clear area. At times it is possible to perceive a filament which binds it to the bud.
Fig. 44. Telophase in which the centrosome is again very distinct.
Figs. 45–48. Subramaniam.
Fig. 45. (S. and Ranganathan, (39), Fig. 1). Early metaphase stage showing the two chromosomes with a developing spindle. At the poles of the spindle could be seen the centrioles surrounded by a clear area representing the centrosphere.
Figs. 46–47. (S., (34), Figs. 31, 32). — Finally one of the two reconstituted nuclei may be seen passing to the bud while the other remains in the mother cell.
Fig. 48. (S., (35), Fig. 4). Cell showing polyploidy ... chromosomes in the cytoplasm are illustrated.
Figs. 49–50. Levan (22), Figs. d, e).
Fig. 49. Normal metaphase in untreated yeast.
Fig. 50. Normal anaphase in untreated yeast.

some other chemical or pipetting a drop of reagent under the cover-slip. The nucleolus appeared consistently in growing cells after treatment with iodine and Sudan black, occasionally with a 1 per cent solution of methyl green.

DISCUSSION

NUCLEAR VACUOLE

A. *Chromosomes.* Our observations show that the yeast cell has the nuclear organization characteristic of other nuclei. Earlier workers are not all in agreement on this point but they have not had the advantages of modern methods, especially the Rafalko Feulgen modification. Janssens and Leblanc (18) were the first to recognize the large vacuole in yeast as the nucleus, and they identified the "dancing body" within the vacuole as the nucleolus (Fig. 27). In Harper's classical paper the fungal nucleus is described with polarized chromosomes attached to the "central body" (centrosome), a permanent cell structure which lies close against the vacuole determining the point of special connection between the nuclear contents and the cytoplasm (Fig. 17). He describes the "strands of chromatin" (chromosomes) as being regularly to the "central body" from which they extend into the central cavity of the nucleus like a cone, with the outer ones sometimes following more closely the inner surface of the nuclear membrane. He describes the "threads" as appearing lumpy or finely granular and of irregular thickness and density since the nodules, which he said "probably consist of chromatin granules in linin", are not of equal size". He states that these threads often have lateral extensions between them, and he describes the free ends of the chromatin threads as frequently being in contact with the nucleolus.

Wager and Peniston (Fig. 22) described the vacuole as containing a loose network of faintly staining threads with numerous deeply stained chromatin granules on the threads. The threads are peripherally disposed and line the interior of the vacuole with the coarser threads clearly showing their connection with the centrosome, which they called the "nucleolus". They state that this network can be seen clearly only under favorable conditions and that the chromatic granules arranged on it are of variable size, sometimes arranged with remarkable regularity but usually distributed unevenly over the network. They state that this network was missed by the majority of workers because they apparently based their conclusions on cells in the early stages of fermentation when the watery vacuolar contents remain unstained in contrast to the cytoplasm, thus making it difficult to see the net-

work. In general, they found that a faintly stained centrosome (our designation) corresponds with a large well stained network, and when the network was small the centrosome was deeply stained. The present authors have found a similar centrosome-chromosome staining relationship with the Feulgen reaction. At an early stage in growth (1–1 ½ hours) the centrosome is Feulgen-positive (Figs. 1–8) and the chromosomes are not visible. After a longer period of incubation (about 6 hours) the chromosomes are Feulgen-positive but the centrosome is not (Fig. 9). Both are rarely Feulgen-positive at the same time.

Lindegren (23) pictured the yeast cell nucleus as similar in structure to that described by Wager and Peniston except that he labelled the structure they called "nucleolus" a centriole. Centrosome is a more general term in its implications and would be preferable. This corresponds with the classical concept of the fungal nucleus.

The majority of yeast cytologists, however, disagree with the above interpretation.

Macallum (27) described the yeast cell as generally containing one large vacuole and frequently a deeply staining extra-vacuolar "corpuscle" surrounded by granules and elongated masses of chromatin. The latter he regarded as identical with the network of fine threads Wager (41) had described in contact with his (Wager's) "nucleolus" (our centrosome). Macallum stated that the "corpuscle" elongates in the process of budding and constricts in the middle, one or both parts passing into the bud. Hoffmeister (16) also described this spherical homogeneous body as the true nucleus (Figs. 19 and 30).

Guilliermond (8, 9) described the same structures seen by Wager (41) but labelled them differently. He insisted that Wager's "nucleolus" was the true nucleus, that it was not always attached to the vacuole, that it possessed the usual nuclear structures such as a nuclear membrane, nucleoplasm and a network that sometimes showed a body like a nucleolus. This "nucleus", he said, divided directly and during budding one portion passed into the bud along with a portion of the vacuole and its contents, which he called metachromatin (volutin), a kind of reserve material. This same spherical body was described by Barker (2) as staining deeply and in well differentiated slides occasionally showing internal structure. Hirschbruch (15), Swellengrebel (40), and Fuhrmann (7) all agreed with Guilliermond that this body is the nucleus, the latter two describing its division in considerable rather accurate detail and interpreting their observation as mitotic division (Figs. 33–38). Janssens and Leblanc and Swellengrebel

figured what we believe similar to a desmose (Figs. 29 and 35), and some of Fuhrmann's figures (Figs. 21 and 37) accurately picture the true chromosomes within the vacuole, though he misinterprets them in his discussion.

Kater (19) described and illustrated, even to showing spindle fibers and sometimes centrioles (Figs. 41 and 42), an almost complete process of mitosis for the centrosome. He stated that the nuclear vacuole was empty. Yet in two of his figures (ex. Fig. 23) he shows a typical fungal nucleus which could be interpreted according to the views of the present authors, with the chromosomes attached to the centrosome and extending into the nuclear vacuole. In the text, however, he disregards this finding and calls the centrosome "a great mass of metachromatic material" and describes the chromosomes as "granular lines of chromatic material in the cytoplasm" *outside* of the vacuole. This error arose from confusing the mitochondria of living cells with the interphase chromosomes of fixed cells (see below). His preferred technique consisted of "fixation in Bouin's and staining before the picric acid was completely washed out. This decreased the affinity of both nuclear and cytoplasmic chromatin material for stain, especially the latter. In this way the nucleus could be stained without showing the chromatic bodies in the cytoplasm at all. This procedure made possible a careful study of the nuclear chromatin both at rest and during division." However, he figured so-called "cytoplasmic chromatic bodies" (Fig. 23) which in our opinion are not in the cytoplasm but are actually the chromosomes in the nuclear vacuole.

According to Badian (1) this yeast "nucleus" is a very primitive structure devoid of membrane and constituted only of two chromosomes which divide longitudinally. Lindegren (25) has shown that genetical analysis demands not less than four chromosomes.

Renaud (33) also described the mitotic process as occurring in the centrosome, which he considered the nucleus (Figs. 24 and 43). He described the internal structure of the centrosome where the chromatin is often arranged in various patterns to give the spurious appearance of mitotic figures (Fig. 7). We have observed the bodies he called centrioles (Fig. 43) as Feulgen-positive granules (Fig. 4). Since he called the centrosome the nucleus it is obvious that he failed to observe chromosomes in the vacuole, which may be because he limited himself to slides prepared with iron haematoxylin. With Heidenhain's, as with most if not all cytological methods, the centrosome takes on the stain with ease, while only after utmost care in differentiation will the contents of the nuclear vacuole appear, especially when the chromosomes are extended in interphase.

Brandt (5) described the centrosome as a "nucleus with two chromosomes". He probably observed the chromatic masses in the centrosome and so considered it the nucleus.

Levan (21, 22) pictured spindles and mitotic phases (Figs. 49–50) in the centrosome. Among his fixatives he lists Carnoy. Feulgen preparations following Carnoy often show arrangements of chromatic material which resemble spindles and chromosomes, and the vacuolar contents often are obscured. Levan considers ten a minimum "chromosome" number from his observations of the centrosomal structure and disagrees with Lindegren's view that this Feulgen-positive body is a centrosome.

Nagel (30) claimed that the nuclear vacuole contained no Feulgen-positive substance, but she tried only the standard Feulgen procedure which usually does not stain small or diffuse chromatin granules and therefore fails to stain the yeast chromosomes. She states that vacuolar granules are lacking in most cases, but that when granules are present in the vacuole they "occasionally appear paired".

Subramaniam and Ranganathan (37) also mistook the centrosome for the nucleus and described "mitosis" of the centrosome, probably because they consistently used Carnoy fixation. Subramaniam later (34) described detailed structures in the centrosome and identified them as nuclear divisions (Figs. 45–47) although his Fig. 6 (Fig. 25) resembles the centrosome with the chromosomes radiating from it into the nuclear vacuole. Like other workers who used the standard Feulgen technique he usually found only the Feulgen-positive centrosome and was less successful in staining the chromosomes in the vacuole. He claimed that what Lindegren described as chromosomes waving in the nuclear sap are in reality mitochondria. A similar confusion of chromosomes in the nuclear vacuole with the mitochondria in the cytoplasm occurred in Kater's (19) paper as well as in Guilliermond's writings. The mitochondria are found in the cytoplasm outside of the nuclear vacuole and are clearly visible in unstained material while the chromosomes generally are not, but the mitochondria invariably disappear from the cytoplasm after treatment with fixatives containing acetic acid. Kater and Subramaniam observed mitochondria in the living cell, destroyed them by fixation, then stained, instead, the chromosomes, and on observation of the stained material assumed that the chromosomes which now appeared were the mitochondria they had seen in living cells.

In recent papers Subramaniam (35, 36) again describes Feulgen-positive bodies which he calls chromosomes. He divides them into two categories a) reconstituted nuclei and b) unorganized nuclei. He considers

the variation in the number of Feulgen-positive particles as evidence of endoploidy (Fig. 36). Since many cells contain no Feulgen-positive particles but presumably contain chromosomes, one must assume that variations in number are not so significant as Subramaniam considers them. It is necessary to search the preparation for cells in which the entire complement of chromosomes is stained. In the study of *S. bayanus* no more than ten chromatinic bodies have been observed in the nuclear vacuole with the Feulgen reaction or with methyl green and usually only two masses in the centrosome, though each of the latter may, on occasion, consist of many granules. This variation in the centrosome from granular to more closely integrated formation makes particle counts relatively meaningless unless the position of the particles in relation to the nuclear vacuole or centrosome is clearly stated, for we have shown that *both* the nuclear vacuole and the centrosome contain Feulgen-positive material.

B. *Nucleolus.* Janssens and Leblanc (18) regarded the central moving granule of the vacuole as the nucleolus and stated that it was always inside the vacuole when present. In their observations of stained material, however, they apparently confused the true nucleolus and the centrosome, calling both the nucleolus. This is evidenced by their figures (Figs. 18 and 26–27) and by the fact that they refer to the nucleolus as a body composed of "nuclein" and describe the divisions of this body at budding (Figs. 28–29).

In Harper's (13) description of the typical fungal nucleus (Fig. 17), he stated that the nucleolus takes on the cytoplasmic stains and that the ends of the chromosomes opposite their attachment to the central body are not infrequently in contact with the nucleolus. He stated further that while the end region of a chromosome might lie on the surface of the nucleolus for a short distance, there was no indication of a fusion of the substance of the two structures, but that the chromosomes appeared to be merely in contact with the nucleolus. Harper drew this conclusion before the relationship of nucleolus to chromosomes had been worked out. In yeasts there is so far no indication that the conventional situation does not obtain.

Wager and Peniston's Fig. 116 (Fig. 22) shows the nucleolus, called "volutin granule" by them, inside the nuclear vacuole in close relationship with the chromosomes. They describe the "characteristic microscopic appearance" of each cell as containing a "large well-defined vacuole with usually one large conspicuous, quickly moving granule towards its center". They also note that this "granule" is always conspicuous after fixation with formalin, Flemming's weak solution, Perenyi's, corrosive sublimate and picric acid. After picric acid they found that,—"The only clearly differentiated

object in the cell is the large granule which in the living condition is observed in the vacuole." In their description of the cell after fixation in Flemming's weak solution they state that the "granule in the vacuole is very conspicuous, is commonly found towards one side of the vacuole and sometimes lodged in a fold" and "occasionally threads connecting with the side of the vacuole show plainly."

CONCLUSIONS

The nuclear vacuole with its attached centrosome and Feulgen-positive chromosomes comprises the nucleus of the yeast cell. The elongated interphase chromosomes extend from the centrosomal area into the nuclear vacuole. In more active cells the chromosomes are rod-shaped, obviously paired, and may lie near the centrosome, in contact with the nucleolus at the inner periphery of the nuclear vacuole. The chromosomes may be found associated with either the centrosome or the nucleolus.

The nucleolus in *S. bayanus* is always single (in contrast to *S. cerevisiae*, Lindegren, (24) and centrally located in the nuclear vacuole and is generally elongate except in very young cells where it is round. The nucleolus almost always takes on cytoplasmic stains except with methyl green and in a few exceptional cases with the Feulgen technique.

The presence of a nuclear membrane is indicated by the regular outline of the nuclear vacuole. The centrosome, always closely associated with the nuclear vacuole, may actually be inside the nuclear membrane, a possibility suggested by the appearance of preparations stained with acid fuchsin. If this be a correct interpretation, it makes understandable the close association of centrosome and chromosome, especially as seen in the interphase nucleus and first recorded by Wager and Peniston. This interpretation is consistent with Harper's statement that, "the connection of the nuclear threads with the central body implies, of course, that the nuclear membrane is not continuous at this point, or at least permits in some way the connection of the threads with the pole."

The centrosome stains purple-red with the Feulgen technique, while the chromosomes stain red-red and the latter often appear somewhat refractile, indicating a possible difference in the nature of the chromatinic make-up of these structures. We propose that the chromatin of the yeast cell be divided into (or called) chromosomal chromatin and centrosomal chromatin. The fact that both do not give a positive Feulgen reaction simultaneously suggests the possibility of a cyclic relationship.

SUMMARY

S. bayanus is an elongate cell whose cytoplasm appears homogeneous in the living state. The nuclear vacuole has the same shape as the cell and contains paired Feulgen-positive chromosomes and a Feulgen-negative nucleolus which is typical in its staining reaction and its relation to the chromosomes. A nuclear membrane is indicated by the definite, regular outline of the nuclear vacuole. When the nuclear vacuole is collapsed the wall lies in folds, establishing the presence of a definite membrane. Certain stains suggest that the centrosome may be within the boundaries of the nuclear membrane.

The centrosome is closely associated with the chromosomes, characteristic of the typical fungal nucleus. The centrosome is Feulgen-positive. The chromosomes and centrosome are not Feulgen-positive simultaneously, suggesting a cyclic relationship. The Feulgen-positive substance of the centrosome when present varies in amount and pattern. It divides, part going into the bud, part remaining in the mother cell. The separating Feulgen-positive masses are connected by a Feulgen-negative strand. The proposal is made that the chromatin of the yeast cell be called chromosomal chromatin and centrosomal chromatin. A *central clear sphere* is present in the centrosome and is closely associated with the Feulgen-positive material.

The cytoplasm contains mitochondria which are more densely aggregated in the area surrounding the nuclear vacuole, the centrosome and the region of the *cytoplasmic clear area*. The mitochondria are visible only in the living cell since the ordinary fixatives destroy them. A *clear area* is almost constantly present in the cytoplasm opposite the centrosome, apparently in contact with the nuclear vacuole.

REFERENCES

1. Badian, J., *Acad. Polon. Bull.* B., **1**, 61 (1937).
2. Barker, B. T. P., *Jour. Fed. Inst. Brewing*, **8**, 26 (1902).
3. Bensley, R. R., and Bensley, S. H., *Handbook of Histological and Cytological Technique*, Chicago, 1938.
4. Beyerinck, M. W., *Cent. Bakt.* I., **16**, 49 (1894).
5. Brandt, K. M., *Protoplasma*, **36**, 77 (1941).
6. Dodge, B. O., *Jour. Agric. Res.*, **35**, 289 (1927).
7. Fuhrmann, F., *Centralbl. f. Bakt.* 2., **15**, 769 (1906).
8. Guilliermond, A. I., *Recherches cytologiques sur les levures et quelques moisissures a formes levures.* Storck, Lyon, 1902.
9. —— *Ann. Mycologie*, **2**, 184 (1904).
10. —— *Centralbl. f. Bakt.* 2., **26**, 576 (1910 a).

11. —— *Compt. Rend. Acad. Sci.*, Paris, **150**, 835 (1910 b).
12. —— *The Yeasts.* (Trans. F. W. Tanner.) New York, 1920.
13. Harper, R. A., *Carnegie Inst. Wash., Publication*, **37**, 105 (1905).
14. Henneberg, W., *Handbuch der Gärungsbakteriologie.* 602 pp. Paul Parey, Berlin, 1926.
15. Hirschbruch, A., *Centralbl. f. Bakt.* 2, **9**, 465, 513, 737 (1902).
16. Hoffmeister, C., Sitzungsb. d. Deutsch. naturw.-med. Ver. f. Böhmen *"Lotus"* in Prague. N. Folge. **20**, 250 (1900).
17. Janssens, F. A., *La Cellule*, **20**, 337 (1902).
18. Janssens, F. A., and Leblanc, A., *La Cellule*, **14**, 201 (1898).
19. Kater, J. Mc. A., *Biol. Bull.*, **52**, 436 (1927).
20. Kudo, R. R., *Protozoology.* Charles C. Thomas. Springfield, Ill., 1947.
21. Levan, A., *Nature*, **158**, 626 (1946).
22. —— *Hereditas*, **33**, 457 (1947).
23. Lindegren, C. C., *Bact. Rev.*, **9**, 111 (1945).
24. —— *Proc. Nat. Acad. Sc.*, **34**, 187 (1948).
25. —— *The Yeast Cell, Its Genetics and Cytology.* Ed. Pub. Inc., St. Louis, 1949.
26. Lindegren, C. C., and Ruman, S., *Jour. of Genetics*, **36**, 395 (1938).
27. Macallum, A. B., *Trans. Canad. Inst.*, **6**, 39 (1899).
28. Marpmann, G., *Centralbl. f. Bakt.* 2., **9**, 357 (1902).
29. McClintock, B., *Am. Jour. Bot.*, **32**, 671 (1945).
30. Nagel, L., *Ann. Mo. Bot. Gar.*, **33**, 249 (1946).
31. Pollister, A. W., and Leuchtenberger, C., *Proc. Nat. Ac. Sci.*, **35**, 111 (1949).
32. Rafalko, J. S., *Stain Tech.*, **21**, 91 (1946).
33. Renaud, J., *Comptes rendus des séances de l'Academie des Sciences*, Paris **206**, 1918 (1938).
34. Subramaniam, M. K., *Proc. Nat. Inst. Sci. India*, **12**, 143 (1946).
35. —— *Curr. Sci.*, **16**, 157 (1947).
36. —— *Proc. Nat. Inst. Sci. India*, **14**, 325 (1948).
37. Subramaniam, M. K., and Ranganathan, B., *Curr. Sci.*, **14**, 78 (1945).
38. —— *Nature*, **157**, 657 (1946).
39. —— *Sc. and Cult.*, **12**, 478 (1947).
40. Swellengrebel, M., *Ann. de l'Institut Pasteur*, **19**, 503 (1905).
41. Wager, H., *Ann. Bot.*, **12**, 499 (1898).
42. Wager, H., and Peniston, A., *Ann. Bot.*, **24**, 45 (1910).

DIFFERENTIATION OF THE SEA URCHIN EGG FOLLOWING REDUCTION OF THE INTERIOR CYTOPLASM IN RELATION TO THE CORTEX

SVEN HÖRSTADIUS, *Zoological Institute, Uppsala, Sweden,*
I. J. LORCH[1] and J. F. DANIELLI[1], *Chester Beatty Research Institute, Royal Cancer Hospital, London, England*

Received October 8, 1949

INTRODUCTION

Ever since the days of Driesch, Herbst, and Boveri the sea urchin egg has been one of the classic objects of experimental embryology. A vast number of results with operative and physiological methods support the notion of Runnström (12) that determination in the sea urchin egg is governed by two principles. These take the form of two gradients along the egg axis with their highest intensity at the animal and vegetal pole. Each gradient extends to the opposite pole, so that they overlap through the whole egg. The gradients are supposed to be of qualitatively different nature, based on differences in metabolism. The existence of these gradients and some of their qualities have been demonstrated by fragmentation and transplantation experiments. Their metabolic characteristics have also been studied in a great number of investigations. See reviews, e.g. by Hörstadius (5, 6), Lindahl (9, 10), Needham (11), Lehmann (7).

It is possible to influence development by chemical means so that either the vegetal or the animal gradient becomes predominant. For instance in the presence of Lithium ions the egg is more or less vegetalized, involving enlargement of the endodermal region at the cost of presumptive ectoderm, reduction of the apical tuft, abnormal development of the skeleton spicules, etc. (3). But the egg can also be more or less animalized, e.g. by action of SCN-ions before fertilization (see 8). Presumptive endoderm becomes ectodermized, and the apical tuft, normally small, extends over a greater part of the surface and skeleton formation may be inhibited. Fragments

[1] Present address: Zoology Department, King's College, London, W. C. 2.

differentiate in a more or less animal or vegetal way, according to their constitution. These fragments, as well as eggs in which different regions have been brought together in varying combinations by means of transplantations (4), show that the two principles have to interact mutually in order to bring about a typical differentiation. At the same time there is evidently an antagonism between them. The one can suppress the other.

As to the localization of the two gradients we have to keep in mind that both extend throughout the whole egg. This is clear from the following facts a) the entire egg can be turned into endoderm or ectoderm by chemical means, b) the most animal material can be turned into endoderm by implantation of micromeres (the most vegetal material), and c) presumptive endoderm can become ectodermized in certain fragments. But are the principles equally represented in the cortex and the interior cytoplasm of the egg, or are they confined to either the surface layer or the interior? Dalcq (2) has suggested that the animal gradient might be cortical, located in the cortex, while the vegetal is in the interior. In view of the fact that reduction of one gradient leads to a preponderance of the other we thought it possible to test this hypothesis by sucking out central cytoplasm before fertilization. In this way the ratio of cortex to central cytoplasm is changed. If a sufficient amount of material representing the one gradient is removed the larva ought to show a shift of differentiation in either direction, first visible in the size of the apical tuft which is the most sensitive indicator.

METHODS AND MATERIAL

Unfertilized eggs of *Psammechinus miliaris* (Gmelin) and *Echinus esculentus* L. were used. The operation was carried out in a paraffin chamber (1) the eggs being contained in a minimum of sea water. The interior cytoplasm was sucked out by means of a micropipette in conjunction with the de Fonbrune micromanipulator. The egg was held in place during the operation by means of a glass hook. This, as well as the pipettes, was made with the aid of the de Fonbrune microforge. Only the more central cytoplasm was removed. The nucleus was left intact. Controls were pierced with the pipette, no suction being applied.

When a considerable amount of cytoplasm was removed the surface of the egg became wrinkled, but turned smooth again within an hour or so. Sperm was added to the drop within 15 min. of the operation. As a rule a fertilization membrane was formed. The eggs were often kept in the paraffin

chamber until the gastrula stage before being transferred to a solid watch-glass.

In order to estimate the amount of cytoplasm removed two methods of measuring the egg diameter were tried. First the circumference of the egg was drawn before and after operation with the egg in the paraffin chamber. This method was not very satisfactory, particularly with the large eggs of *Echinus esculentus*, because compression of the egg in the small drop is unavoidable. Thus the visible diameter of the egg is no longer a true measure of its volume, computed as a sphere. The degree of flattening appeared to be greater after cytoplasmic reduction, hence the estimates of the amount of cytoplasm removed, based on egg diameter, are probably too low. The second method, which was finally adopted, involved drawing the egg after this had been lying free in a larger amount of sea water for some time. The diameter of these eggs was compared with the mean size of ten eggs from the same female drawn under similar conditions, since for technical reasons it was not possible to draw each egg before placing it in the paraffin chamber (i.e. before compression).

RESULTS

Of 35 operated *Echinus esculentus* eggs ten reached the prism or pluteus stage. Some of these belonged to preliminary experiments when no measurement of size was made. Four eggs which subsequently developed into plutei were measured by the first method. Their volumes corresponded to 73.5, 74, 75 and 79 per cent of the original egg volume. The volumes of the first two eggs were also estimated according to the second method and the result was 48 per cent for both. This is likely to be the more reliable figure. The size relation of eggs before and after a reduction of this degree is shown in Fig. 1 A.

In some cases an attempt was made to measure the size of the small gastrulae in relation to normal ones. The swimming gastrulae sometimes rotate on the same spot with the apical tuft towards the coverslip. The equatorial circumference of two gastrulae could thus be drawn with the camera lucida. The size of one "reduced" gastrula in relation to a normal one is shown diagrammatically in Fig. 1 B. If the volume as a sphere is calculated the two small gastrulae measured represented only 22 and 31 per cent resp.

The development of the healthy larvae was normal in every respect. The apical organ was of typical size. No deviation in either animal or vegetal

Fig. 1. A_1 & A_2 Normal and reduced eggs of *E. esculentus*. B_1 & B_2 Gastrula of *E. esculentus* developed from normal and reduced egg resp.

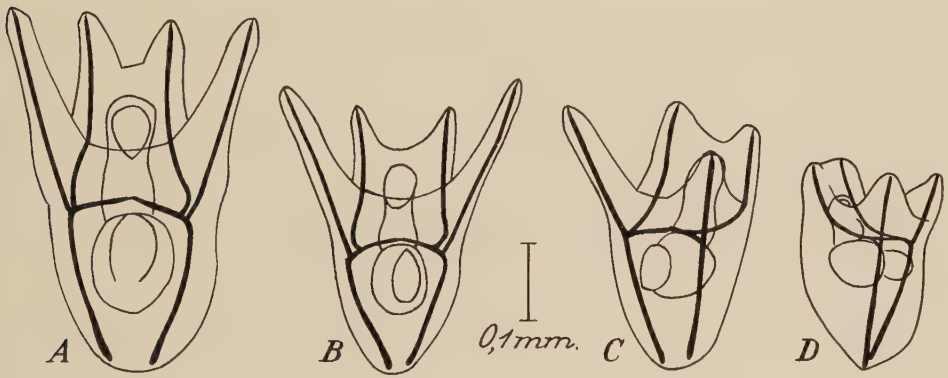


Fig. 2. Plutei of *Echinus esculentus*. A. Normal pluteus. B–D. Plutei developed from eggs with reduced cytoplasm. Degree of reduction of volume expressed as per cent of original volume, B 26 per cent (1st Method) 51.1 per cent (2nd Method), C 21 per cent (1st Method), D 25 per cent (1st Method) 69 per cent (gastrulae measured).

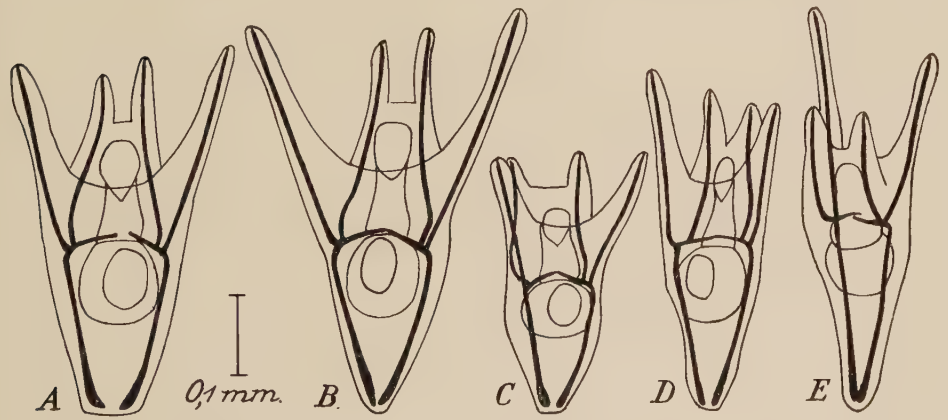


Fig. 3. Plutei of *Psammechinus miliaris*. A & B Normal plutei. C, D, E. Plutei developed from eggs with reduced cytoplasm. Degree of reduction of volume expressed as per cent of original volume estimated by 1st Method C 56 per cent, D 20 per cent, E 40 per cent.

direction was observed. Fig. 2 shows three of the "reduced" plutei (B – D) together with a normal larva (A).

Of 25 operated eggs of *Psammechinus miliaris* ten started development. Of these one was lost, two did not get beyond the early gastrula stage and the remaining seven developed into plutei. The volumes of the reduced eggs were 81, 80, 70, 61, 60 and 44 per cent of the original, as estimated by the first method. Fig. 3 shows three of the small plutei compared with two normal ones.

DISCUSSION

Although our material is not extensive it seems to us sufficient to answer our question. The interior cytoplasm has been considerably reduced in 20 eggs which afterwards started development. In at least 3 eggs the reduction amounted to more than 50 per cent. The same was probably also the case in some of the other eggs, with which the method used to calculate the volume did not do justice to the degree of reduction. We have therefore considerably changed the ratio of cortical to central cytoplasm. This had no effect on the mode of differentiation. Hence it is impossible that one of the two acting principles is confined to the cortex, and the other to the interior cytoplasm.

It may be thought that our results confirm the idea that the surface layer is of predominant importance in determination. The interior cytoplasm has been to a great extent removed. As a consequence the remaining cytoplasm must have been more or less deranged, and yet normal development ensued. But such a conclusion is not justified from these experiments. The typical development may be a consequence of the unaltered conditions in the cortex that plays such a commanding role, but this is by no means proved by this experiment. We must also assume that a regulation can take place in the disarranged interior cytoplasm, leading to typical development. This assumption is supported by the fact that small and well proportioned plutei arise e.g. if the equatorial part of an egg is omitted, i.e. after fusing the most animal with the most vegetal material (4).

This work was carried out in March and April 1949 at the Marine Biological Laboratory, Plymouth. We are greatly indebted to the Director, Dr. F. S. Russell, and his staff for facilitating our work in every way. S. Hörstadius is indebted to the British Council and the Swedish Research Council for grants. The balance of expenses was defrayed by the Royal Cancer Hospital.

SUMMARY

1. The central cytoplasm of eggs of *Echinus esculentus* L. and *Psammechinus miliaris* (Gmelin) was reduced by suction before fertilization. The total cytoplasmic reduction amounted to about 50 per cent.

2. In 20 eggs which started development no deviations in either animal or vegetal direction were observed. The resulting plutei were normal in every respect except size.

3. The results are contrary to Dalcq's concept of the differential localization of gradients at the centre and periphery of the sea urchin egg.

REFERENCES

1. Commandon, J. and de Fonbrune, P., *Ann. de L'Inst. Pasteur*, **60**, 113 (1938).
2. Dalcq, A., *L'œuf et son dynamisme organisateur*, Paris, 1941.
3. Herbst, C., *Z. wiss. Zool.*, **55**, 446 (1892).
4. Hörstadius, S., *Pubbl. Staz. Zool. Napoli*, **14**, 251 (1935).
5. —— *Biol. Reviews*, **14**, 132 (1939).
6. —— *Pubbl. Staz. Zool. Napoli*, **21**, 131 (1949).
7. Lehmann, F. E., *Einführung in die physiologische Embryologie*, Basel, 1945.
8. Lindahl, P. E., *Acta zool.*, **17**, 179 (1936).
9. —— *Die Naturwissenschaften*, **29**, 673 (1941).
10. —— *Quart. Rev. of Biol.*, **17**, 213 (1942).
11. Needham, J., *Biochemistry and Morphogenesis*, Cambridge, 1942.
12. Runnström, J., *Roux Arch. Entw. Mech. Organ.*, **113**, 556 (1928).

THE FINE STRUCTURE OF MYOFIBRILS

G. ROZSA[1], A. SZENT-GYÖRGYI[1], and RALPH W. G. WYCKOFF

Laboratory of Physical Biology, Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Maryland

Received October 25, 1949

As part of a more extensive series of electron microscopic observations on muscle and muscle proteins we have recently described phenomena observed in connection with the polymerization of actin (11). It was there shown that when globular actin is transformed directly on the electron microscopic substrate, the resulting fibrous actin appears either as a regular two dimensional network of cylindrical units or as threads which presumably are end-to-end associations of these units. Such actin threads closely resemble in size and appearance the filaments that are conspicuous within the myofibrils of vertebrate muscle. The present paper is devoted to electron micrographic descriptions of such myofibrils showing these filaments and other macromolecular elements occurring in the muscle fibers and their sarcolemmatous membranes.

All the experiments to be described were carried out on rabbit muscle. To make preparations for electron microscopy the middle part of the *musculus psoas* was separated into thin bundles *in situ*, each of which was fixed in its resting state by being tied at either end with coarse thread to an applicator or similar thin strip of wood. These attached bundles were then cut from the muscle and placed with their wooden supports in cold distilled water or 0.2 M KCl solution, or sometimes in 5 per cent formalin solution, and stored at 0° C. If formalin was used, the fixative was washed out very carefully after half an hour. No essential difference could be seen between fresh (unfixed) muscle and muscle fixed in this way. When preparations were to be made, the muscle was removed from the wood, broken up by agitation in the Waring blendor; or, when this method proved too drastic, it was teased apart by needles. For examination these separated muscle

[1] Special fellows, National Institutes of Health, U. S. Public Health Service.

fibrils, with or without additional treatment, were mounted on Formvar substrates and shadowed with palladium.

In the first series of experiments attention was directed towards the fine structure of the myofibrils themselves. At low magnification shadowed individual myofibrils have had the general appearance of Fig. 1. The bands as usually designated can be recognized through the keys appended to this and Fig. 3. Thus the anisotropic (A) and isotropic (I) bands are clearly seen with their bisecting M and Z bands and H "disks", the last being in the middle part of the A band on each side of the M band. The N band can be observed in Fig. 3 which shows a myofibril at a higher magnification; but here the H disks are absent. Our photographs support the findings of Hall, Jakus and Schmitt (6) and conflict with those of Pease and Baker (9) in always showing a well-defined differentiation of the foregoing bands. Even in unfixed material the demarkation of the different striations has been sharp. We have never failed to find the Z and M bands and have usually found the H disks in rabbit muscle. The N bands can easily be removed by the washing needed to remove salt and, accordingly, have often been absent from the finished preparations. Pease and Baker (9) have stated that vacuoles are to be seen between the filaments after removal of these N bands. We have, however, been unable to confirm this and have never found vacuoles, other than obvious tears, between filaments even at the far higher magnifications we have used.

Fig. 1 illustrates the fact, made still clearer in subsequent photographs, that the myofibrils are made up of closely packed thin filaments which run continuously through the bands. It is important that this packing does not appear altered by removal of the bands and is everywhere the same along the myofibrils. From their shadows it is evident that the A, Z and M bands are much thicker than the isotropic bands and H "disks." They, therefore represent regions where an additional material is present along with the longitudinal filaments. It is generally agreed that most of the ash of incinerated muscle is localized in the regions of the A bands. Hall, Jakus and Schmitt (6) have suggested that the greater electronic opacity of these bands is due to the potassium bound in them while Draper and Hodge (4) have stated that "a regular fine transverse striation is present arising from a periodic concentration of magnesium and calcium bound to the actomyosin framework." Complete verification of this last statement will be difficult. It seems clear from our electron micrographs that more than the inorganic salts are concentrated in the dense striations. This additional material, presumably protein and perhaps rich in salts, overlies the fila-

mentous body of the myofibrils and perhaps is also interspersed between these filaments. On the contrary, the H "disks" and the isotropic bands appear as deficient in this substance. These demonstrations that the "A substance" and the similar substances distributed in the Z, M and N bands are largely extrafibrillar are supported by the fact that the striations can all be washed away without disrupting the myofibril as a whole or sensibly disturbing its filaments (Fig. 2).

Pease and Baker (9) have considered that "the cores of the myofibrils were essentially hollow except insofar as they were longitudinally compartmentalized" and that Hall, Jakus and Schmitt "may have occasionally isolated length of walls of myofibrils" only a single layer thick for their observations. Recently Draper and Hodge (4) have made the somewhat similar suggestion that "the myofibrils are thin sheets of fibrous material, which in the intact muscle are folded about the long axis and embedded in bundles of collagen protofibrils." The present electron micrographs do not confirm either of these points of view. They do not indicate that the myofibrils are tubes which are occasionally opened up or that they are thin sheets folded about the long axis. Although collagen fibers are found in muscle tissue, none of our preparations has indicated that the myofibrils are embedded in bundles of collagen protofibrils.

The structures seen in the electron microscope reopen the important question of the correct interpretation to be given the details of muscle structure seen under the polarizing microscope. Barer (1) has stated that "the filaments tend to be rather better oriented in the A band than in the I band," presumably with the implication that the observed double refraction is primarily due to filament orientation. Anisotropy of muscle is to be expected from the strong positive birefringence of both F-actin and myosin, but our failure to find a difference in filament orientation between isotropic and anisotropic regions shows that the optical dissimilarity of the two regions cannot arise from arrangement of the filaments. Furthermore, the second material in the A band would tend to reduce the birefringence of its filaments compared with those in the isotropic region. There seem two possible ways out of this difficulty. One is to consider that the "amorphous" material of the A bands has optical properties such that it rather than the filaments is responsible for the anisotropy of the muscle fiber; or to assume (3, 8) that a negative birefringent substance exists in the isotropic region. A second way out of the difficulty would be found if the commonly accepted nomenclature should correspond to a mistaken correlation between the optical microscopic and the electron microscopic detail. While this may

seem improbable, it must be remembered that the bands are not always easy to see in the polarizing microscope and that apparent shifts of the striations commonly occur when focusing and shifting from ordinary optical to polarizing microscopic observations. A direct photographic comparison of the polarizing microscopic and electron microscopic images of the same myofibril should be made to remove this uncertainty.

Many electron micrographs of intact myofibrils give evidence of a finer structure regularly repeated along the axis of the fibril. Hall, Jakus and Schmitt (6) and Draper and Hodge (4) have described this as a periodicity of 400 A along the myofibrils with the implication that this periodicity represents the finer structure of the filaments themselves. In our shadowed photographs it appears (Fig. 4) rather as a series of very closely spaced bands which like the greater bands described above are produced by structure and material superimposed on the filaments. This 400 A periodicity is best seen in the thinner H and I regions. Its frequent absence from these regions is perhaps to be explained by the fact that, like the N bands, it is readily removed by washing.

In a second series of experiments we have studied more closely the filaments that constitute the main bulk of the myofibrils. As is clear from Fig. 5–7 they can be especially well seen in partially disintegrated fibrils. Perhaps their most striking feature is the uniformity of their cross sections. No variation can be detected in a constant width of about 100 A. In this our results conflict with those of Hall, Jakus and Schmitt (6) who found diameters varying between 50 and 250 A. Such an apparent non-uniformity in filament diameter may have been due in part to the staining used and in part to a lower contrast that did not provide a sharp discrimination between single filaments and bundles of a few filaments.

The length of the filaments seen in our photographs varies greatly due to the technique of preparation. It has not been possible to prepare them separate from one another without mechanical agitation, and they have accordingly broken readily into shorter pieces. This break has usually occurred at the edge of a band and has resulted in a preponderance of filaments half a sarcomer, or ca one micron, long. But many filaments are three or four micra long; and they offer additional direct evidence that the filaments run continuously through the sarcomers. Fig. 7 shows a loosened segment of the myofibril with the remains of a band bisecting it. It is not easy to decide whether this segment corresponds to the I or A band, but the picture demonstrates that the bisecting band, whether Z or M, does not disturb the continuity of the filaments that passed through it. In most pre-

parations the filaments have been covered by a material whose fine structure has interfered with observations of the finer structure of the filaments themselves. The only evidence for such a finer structure has appeared on occasional clear places found sometimes within a myofibril and sometimes on isolated filaments. These have consisted of a beaded structure with a periodicity that has averaged between ca 250 A and 300 A. Even in these favorable instances detail has not been sufficiently clear to show with certainty whether it is to be identified with remains of the 400 A micro bands described above or with a segmentation of the filaments themselves.

It should be mentioned that Bennett (2) has stated that the filaments in myofibrils show a helical or coiled spring-like ultrastructure. The coiled thread within the filament is said to be less than 50 A in diameter and about 6 turns are said to be discernible within an overall periodicity of ca 400 A. No photographs we have made contain any evidence for such an ultrastructure.

The whole appearance of the filaments is compatible with the hypothesis that they are polymerized actin threads covered by some second substance of unresolved structure. Electron microscopy has not yet shown the whereabouts of the myosin that is present in myofibrils, but it is possible that it may be this unresolved material. Electron micrographs we have recently obtained of crystallized myosin show uniform, exceedingly fine threads that presumably are its molecules (12). Possibly the filaments shown in the electron micrographs of this paper are lengthwise associations of such myosin threads with the thicker threads of F-actin.

In a third series of experiments we have sought electron microscopic evidence concerning the nature of the sarcolemma. Unfortunately, there has been considerable confusion in identifying this structure. Jones and Barer (7) have described it as a membrane devoid of visible filaments but characterized by numerous spots 400—1,000 A in diameter. Pease and Baker (9) also consider it as a membrane that has no resolvable structure but contains cementing substances.

To photograph sarcolemma we have isolated single muscle fibers and opened the macroscopic sarcolemma mechanically. In preparations made from them the whole content of the muscle fiber could be observed; myofibrils, singly or in bundles, completely isolated cell nuclei and very large thin sheets. If we have seen sarcolemma it is in the form of these sheets. Fig. 8 shows part of such a sheet at low magnification. It has a granular surface but no other obvious fine structure. Folds such as that seen in Fig. 9 indicate that it is a real membrane and not merely a thin deposit

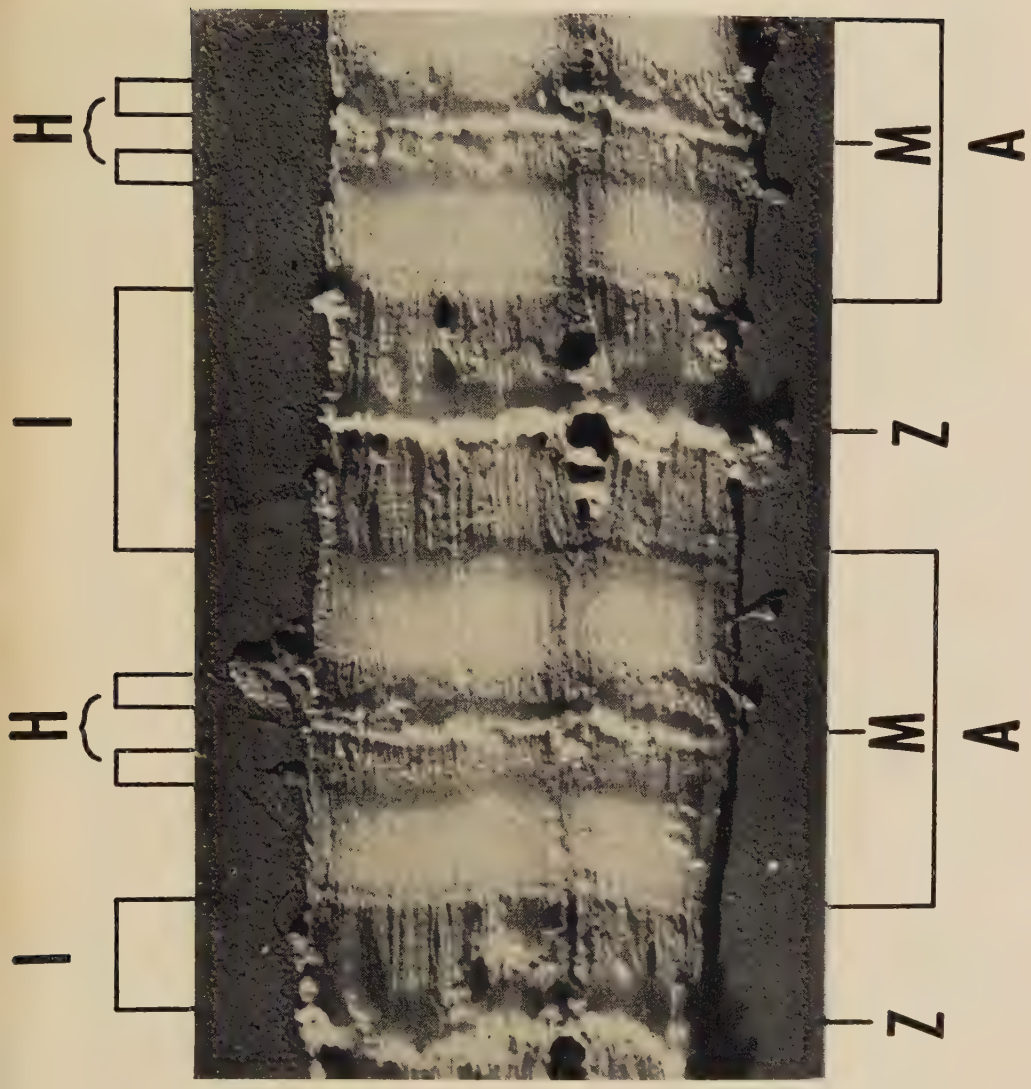
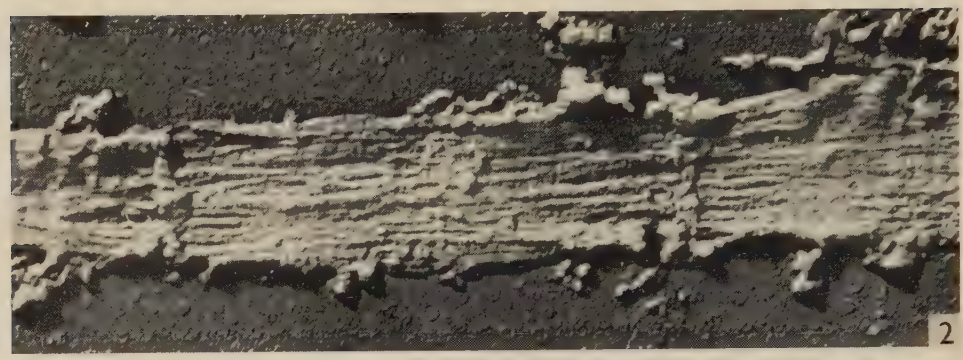


Fig. 1. An electron micrograph of a portion of a myofibril from the musculus psoas of a rabbit. The several bands that have been described in this striated muscle are marked along the side of the photograph. Palladium shadowing. Magnification = 30,000 X.



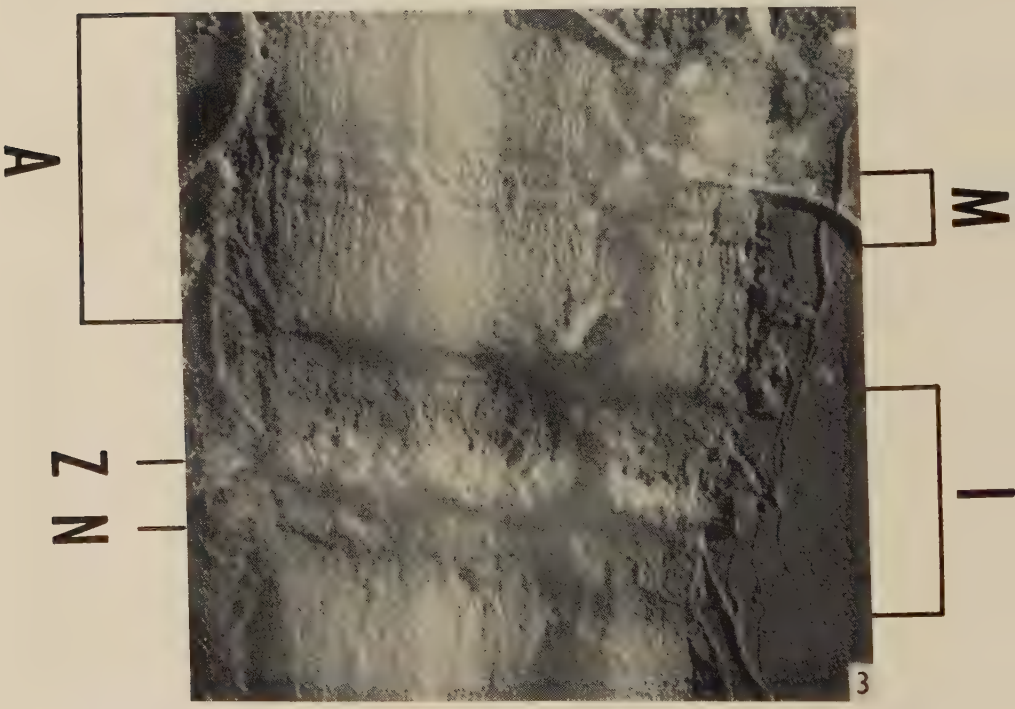
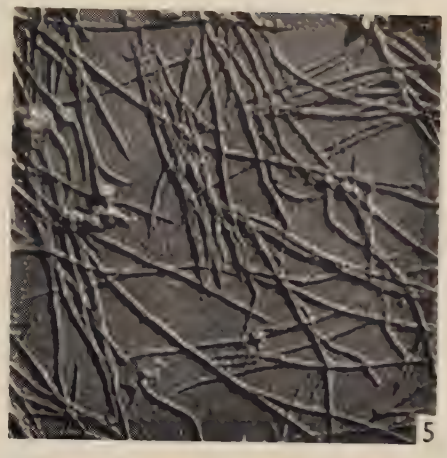


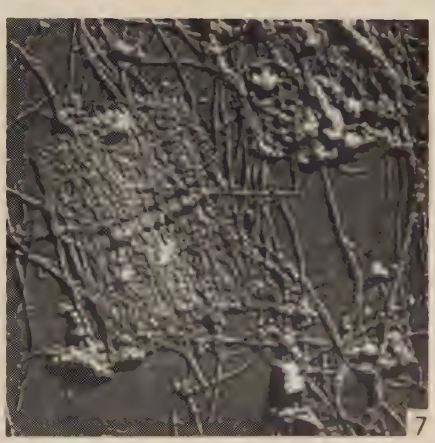
Fig. 2. A portion of a myofibril from which almost all traces of the bands have been removed by washing. Magnification = 30,000 X.

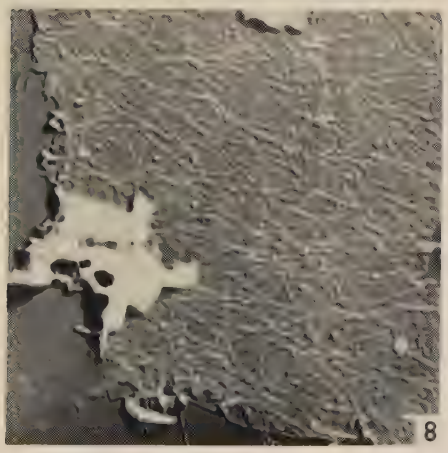
Fig. 3. An electron micrograph of part of a similar myofibril. Here too bands are given their conventional designations. Palladium shadowing. Magnification = 32,000 X.

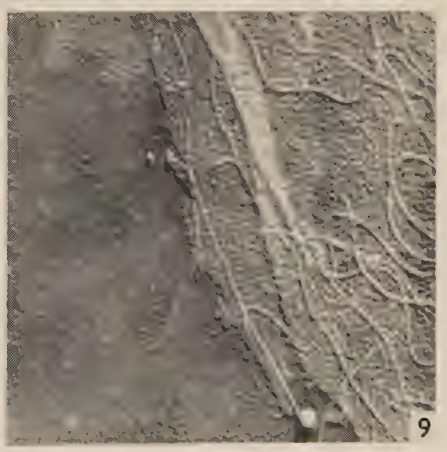
4


5

6


7


8

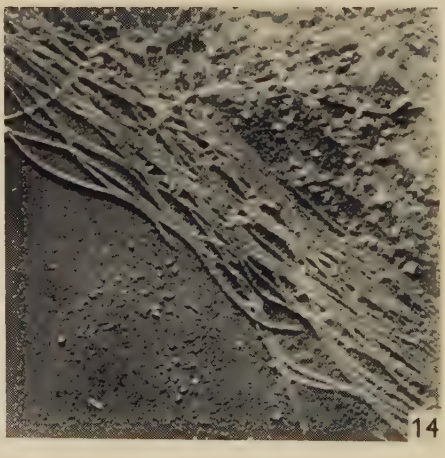

9

10

11

12

13



14

from some soluble salt or protein extracted from the broken myofibril. Thick threads like those to be seen in Fig. 9 are usually found associated with these membranes. Though our photographs do not demonstrate the fact beyond question, the threads apparently are not an integral part of the body of the membrane but are attached to its surface and form part of a system of threads surrounding the muscle fibers.

These threads are very long and have a well-defined fine structure visible even at low magnifications. It is best seen in preparations that have been cleaned by washings carried out at a slightly alkaline pH. Often after such treatment the threads are found in great masses and networks (Fig. 10). Examination at higher magnifications (Figs. 11–13) reveal marked differences and similarities between them and the conventional fibers of collagen. The collagen fibers of tendon have indefinite and often very considerable widths; on the contrary the threads shown here have an essentially uniform width of only ca 250 A. They occur in bundles, as do the collagen fibrils of tendon, but Figs. 12 and 13 show that their cross-striations are usually continuously repeated rather than in the paired fashion that yields the collagen disks 650 A apart. Recent work (10) has, however, demonstrated connective tissue fibrils that are sometimes continuously cross-striated and that sometimes show both continuous and interrupted cross-striation in the same

---

Fig. 4. A portion of a myofibril showing, especially within the isotropic band, a sequence of very fine band-like structures about 400 A apart. Magnification = 25,000 X.

Fig. 5. This and the following two electron micrographs show the filaments of a myofibril band more or less completely dispersed. Some of the separated filaments show remnants of an original "beaded" cross structure; otherwise, they appear homogeneous. Magnification = 28,000 X.

Fig. 6. The filaments of this photograph are not so dispersed that their original association has been completely lost. Magnification = 28,000 X.

Fig. 7. The filaments here are scarcely disturbed from their arrangement to form a band of the original myofibril. Magnification = 25,000 X.

Fig. 8. A membranous sheet with associated bundles of fibers which presumably is to be identified with sarcolemma. Magnification = 9,000 X.

Fig. 9. Another piece of sarcolemmatous membrane showing more clearly the associated fibers. Note the granular texture of the membrane and the vertical fold in it. Magnification = 12,000 X.

Fig. 10. A dense mesh of the fibers seen after washing a membrane at a slightly alkaline pH. Magnification = 8,000 X.

Fig. 11. The striated fine structure of these fibers is readily visible at the higher magnifications of this and following photographs. Magnification = 25,000 X.

Fig. 12. Often these fibers are found in closely knit associations in which the continuous cross striations of neighboring fibers are well aligned. Magnification = 32,000 X.

Fig. 13. The relative diameters of these fibers and of the filaments of the myofibrils are clearly shown in this photograph. Magnification = 25,000 X.

Fig. 14. Sometimes the fibers associated in sarcolemmatous membrane are continuously cross striated, as in the preceding photographs; sometimes as in this photograph they have the regularly interrupted system of striations typical of classical collagen. Magnification = 20,000 X.

fibril. We have seen a collagen-like interrupted striation in occasional groups of the thin uniform fibrils shown in this paper (Fig. 14).[1] In both kinds of continuously striated threads the fundamental separation between striae is about 200 A. Evidently much further work is needed to define adequately the relation between the two fibers and the fundamental character of the striae of each. Two possibilities can be imagined depending on whether the cross structure is the remains of a system of threads binding the filaments together or is a fine structure within the filament themselves. Evidence has been presented (10) which suggests that the cross striations of collagen may be of the first sort. The general appearance and uniform diameter of the muscle-associated threads described here, on the other hand, make it conceivable that they might be side-by-side associations of flattened cylindrical elements.

If this were the case there would then be an analogy between their structure and that of the F-actin threads which, according to our previous work (11), are associations of elongated cylindrical units. In the case of actin the units are about 100 A in diameter and 300 A long. If units should exist in the threads of Figs. 11–14, their dimensions would be ca 250×200 A. The relative sizes of these cross-striated threads and the actin-like filaments of the myofibrils is especially well brought out in Fig. 13 where they lie adjacent to one another.

A continued study of their fine structure is needed to provide an understanding of the function of these striated threads. Are they and the sarcolemmatous membrane with which they are associated essentially a framework and support for the muscle fibers or do they have properties of extensibility which give them an essential role in determining the physical state of muscle? Our electron micrographs have demonstrated (11) that the units of F-actin are capable of both an end-to-end and a more or less side-by-side association. If the striated threads shown above should prove to be similar associations of cylindrical units, their appearance might be expected to change with the state of the muscle to which they belonged. This possible extensibility of the threads also raises the question of whether they may be related to fibers of yellow elastic tissue. They show none of the spiral structure which Gross (5) has recently described for the elements of such tissue, but they do resemble fibers that have been photographed from elastic tissue that has been subjected to prolonged tryptic digestion (10).

[1] Reed, R. and Rudall, K. M. (Biochim. Biophys. Acta **2**, 19 (1948)) described a network of typical collagen fibers surrounding the muscle fibers. The fibers they show, which presumably are part of the connective tissue system holding together the muscle fibers themselves, appear different from all the fibers shown here except possibly those of Fig. 14.

SUMMARY

Shadowed electron micrographs show that myofibrils of rabbit striated muscle consist of bundles of long filaments overlaid and perhaps interpenetrated by a structureless material whose distribution determines the banded character of the muscle. The filaments have about the diameter of those of fibrous actin. The photographs show many details of the fine structure of the myofibrils and their components as well as of the sarcolemma and the thicker fibers associated with this membrane.

REFERENCES

1. Barer, R., *Biol. Rev.*, **23**, 159 (1948).
2. Bennett, H. S., *Anat. Rec.*, **103**, 423 (1949).
3. Dempsey, E. W., Wislocki, G. B., and Singer, M., *Anat. Rec.*, **96**, 221 (1946).
4. Draper, M. H., and Hodge, A. J., *Nature*, **163**, 576 (1949).
5. Gross, J., *J. Exp. Med.*, **89**, 699 (1949).
6. Hall, C. E., Jakus, M. A., and Schmitt, F. O., *Biol. Bull.*, **90**, 32 (1946).
7. Jones, W. M., and Barer, R., *Nature*, **161**, 1012 (1948).
8. Matoltsy, A. G., and Gerendás, M., *Nature*, **159**, 502 (1947).
9. Pease, D. C., and Baker, R. F., *Am. J. Anat.*, **84**, 175 (1949).
10. Pratt, A., and Wyckoff, R. W. G., *Biochim. Biophys. Acta.*, **5**, 1 (1950).
11. Rozsa, G., Szent-Györgyi, A., and Wyckoff, R. W. G., *Biochim. Biophys. Acta*, **3**, 561 (1949).
12. —— (Unpublished data of the authors.)

THE BASIC DYE-UPTAKE AND THE PRESENCE OF A GROWTH-INHIBITING SUBSTANCE IN THE HEALING TISSUE OF SKIN WOUNDS

A. BALAZS and HJ. HOLMGREN

Department of Experimental Histology, Karolinska Institutet, Stockholm

Received October 12, 1949

The process of wound healing has long attracted a great deal of interest. Detailed studies have been made of e.g. the histologic picture, the tensile strength of healing wound (18), the appearance of so-called wound hormones. In the present communication it has not been considered necessary to discuss these investigations, as they have already been extensively reviewed (1).

The present work deals with the problem, whether any regulative factor occurs in the growing tissue of the wound, which alters the undifferentiated growing tissue to differentiated tissue.

There is a metachromatic change in growing tissue caused by high molecular, acid polysaccharides (2, 6, 7, 8, 17, 18). Therefore, in order to illustrate wound-tissue alterations from another viewpoint, we have investigated the variations in this tissue's ability to bind the basic dye.

MATERIAL AND METHODS

In the experiments white male rats were used, weighing about 150–200 g and in each series the animals used were of the same size.

Under sterile precautions a wound about 5 cm in length was cut on the back of the animal and sutured with silk. After varying periods of time the animals were killed, and the wounds were cut out with as little skin as possible. The wound tissue was then examined according to the following method.

1. *The basic dye binding reaction*

In an ordinary freezing microtome the wound tissue was cut into sections, 100–200 μ in thickness. It should be pointed out that carbon dioxide snow must not come into contact with the preparations, as it disturbs the adsorption of dye. The weight of the sections was determined in constant moisture with an accuracy of within 0.2 mg.

The sections were kept in 1 mg per cent of toluidine blue dissolved in water, for 24 hours at +2°—+4° C, whereupon the absorption of light was determined in a control solution and in the staining solution with which the preparation had been treated. The amount of dye adsorbed per mg of fresh tissue was determined from the difference. The determinations were made by the aid of a photoelectric colorimeter at 6,000 Å.

2. *Methods for the preparation of the extracts*

a) The frozen tissue with as little normal skin as possible was cut in a freezing microtome in sections 200–300 μ in thickness. In this way a few grams of fresh tissue were obtained from each wound. One portion of the finely divided tissue was then extracted for 24 hours in distilled water, another portion in physiological saline solution, both at +4° C. The extracts were dialyzed in water for 24 hours. Both solutions were diluted in order to give an equal quantity of the original material per unit of volume. The solution was sterilized with Seitz bacterial filter.

b) The effect of the extracts on the cell growth was investigated in the following manner:

Heart fibroblasts from 12–14 day old chickembryos were grown on adult chicken plasma in Carrel flasks. In order to secure as pure growth conditions as possible, embryonic extract was not used but the plasma was left to coagulate slowly by itself. After 24 hours the growth was determined by the planimetric method and the fluid phase was added. The extracts to be examined were added to the liquid medium in equal concentrations. The liquid medium of the control cultures was physiological saline solution.

The growth of the culture was determined during four days after the addition of the extracts. At the same time the growth of the control cultures was ascertained. The growth of the experimental cultures was then determined in relation to the growth of the control cultures.

3. *Histological methods*

a) Staining with toluidine blue in order to examine the granulation tissue for metachromasia (frozen sections).

b) Argyrophilic fibrils according to Gomori's method.

c) Alkaline and acid phosphatase according to Gomori.

d) Staining with azan according to Heidenhain.

RESULTS

Figs. 1–5 show the results obtained partly from determining the power of the tissue to combine with basic dyes, partly from experiments to ascertain the effect of the extracts on the growth of the tissue culture.

The experiments include three different series. The first and the second series are complete, the third series, however, is incomplete, as we did not follow the changes in the granulation tissue for more than seven days after the wound was made.

1. *Investigations on the power of the granulation tissue to combine with dye substance*

The amount of bound dye in γ per mg of tissue varies for normal skin between 2.0 and 3.0 γ. After the wound has been made this value decreases even during the first hour to about 1.5 γ. The decline is probably due to bleeding and loss of fluid; it can in any case not be attributed to new growth of cellular components. During the next few days the values range from 1.0 to 3.0 γ. They do not rise until the fourth day. Maximum is reached on the sixth to eighth day after making the wound, when the values for bound dye lie somewhere around 6.0 γ per mg of tissue. After another slow decline, the values seem to become normal on the 15th day. Finally, after a month, the values range between 2.0 and 3.0 γ.

In a fourth series we extracted the frozen sections with water for 24 hours at $+2°$—$+4°$ C before determining the amount of dye-uptake. The purpose was to separate the components that may bind the dyes and cause the above described variations in the effect. As seen from Fig. 1 the values for all the preparations treated in this way lie between 1.0 and 2.0 γ. The same values are recorded on similar treatment of normal skin (see Fig. 1). The variations observed in the other series must thus be due to some other sub-

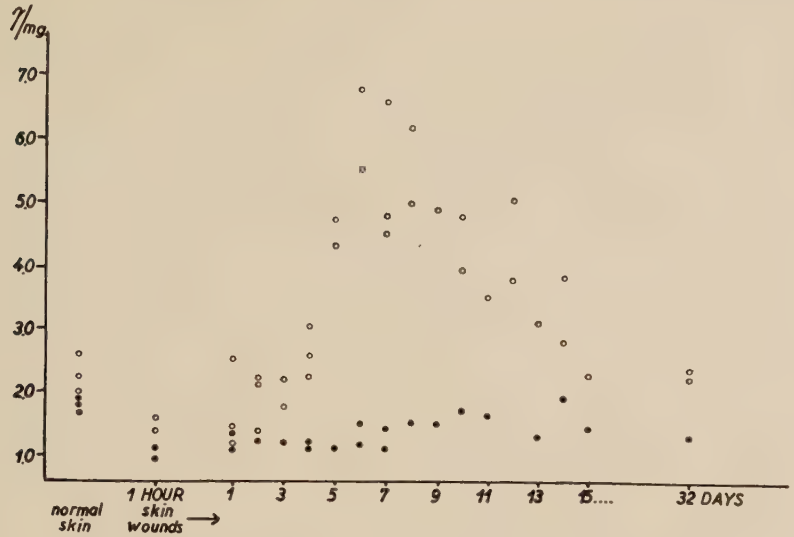


Fig. 1. The dye binding power of healing tissue of skin wounds.
O before water extraction • after water extraction.

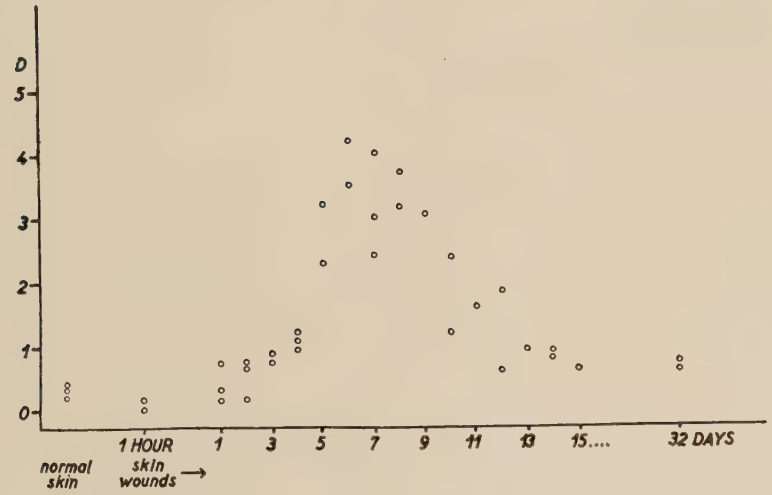


Fig. 2. The dye binding index (D) plotted against days of wound healing.

$$D = \frac{F - Fw}{Fw}$$

F = The uptake dye per mg fresh tissue
Fw = The uptake dye per mg fresh tissue after water extraction.

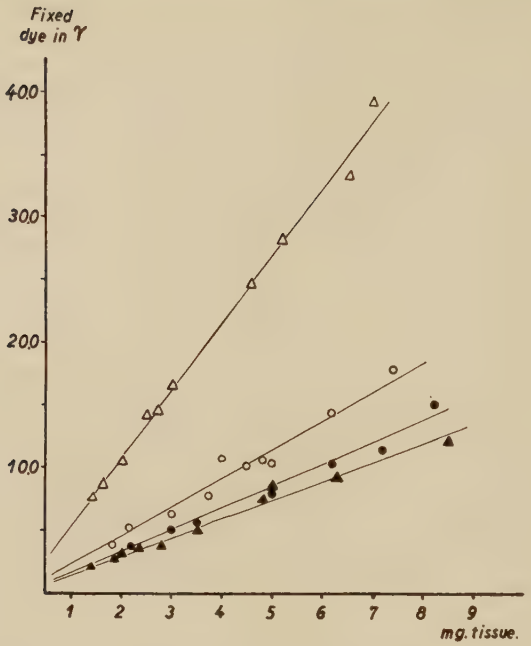


Fig. 3. The dye binding plotted against different quantity of fresh tissue.
△ Wound tissue (6 days)
○ Normal skin.
▲ Wound tissue after water extraction (6 days)
● Normal skin after water extraction.

stances, relatively easily soluble in water, as the dye-uptake after water extraction varies only slightly.

In order to demonstrate further the variation in the capacity of the wound to take up basic dyes and to obtain a clearer picture of the variations we have calculated an index representing the true value for the uptake and the corresponding value after water extraction (Fig. 2). As the process is largely identical to that illustrated in Fig. 1, a discussion of the diagram is superfluous.

When the size of the tissue sections varies from one experiment to another it is naturally important to ascertain whether the uptake of dye varies systematically in relation to the amount of tissue used on each occasion.

The results of experiments with tissue sections varying from 1.5–8.5 mg are recorded in Fig. 3. As seen in the diagram, the uptake of dyestuff is on the whole directly proportional to the weight of the examined prepa-

rations. The tissue sections used in these experiments were of varying thickness (100–200 μ) and were spread out to allow the dye to diffuse properly. The curves for untreated normal skin and for normal skin extracted with water almost coincide; the former curve is slightly steeper than the latter, the difference in slope between the curves for untreated normal skin and for normal skin extracted with water largely falls within the margin of errors. The curve for the wound tissue shows a markedly steeper slope and differs altogether from the curve for normal skin. After water extraction of skin from six day old wounds there is a considerable fall in the values and the curve coincides with the corresponding curve for normal skin.

The results seem to prove that reliable values are obtained by this method despite the variations in the weight of the examined preparations. The curves show that the uptake of dye is directly related to the absolute weight of the tissue.

2. *Investigations on the cell growth in vitro in the presence of wound extracts*

The growth of the fibroblast cultures with or without the addition of wound extract is recorded graphically in Figs. 4 and 5. In Fig. 4 the growth of the normal cultures in plasma is represented by a wide horizontal band. The normal variations in the growth of the control cultures range within this area.

The growth of the experimental cultures was estimated in relation to the growth of the control cultures. In Fig. 4, e.g. +20 per cent and −20 per cent indicate that the cultures concerned have grown 20 per cent more and 20 per cent less, respectively, than the controls.

As seen from the diagram the cell growth increases when cultures are grown on a medium with the salt-soluble factor (globulin) and that this growth-increasing effect is more marked if extracts from one to two days old wounds are used. After this the effect of the salt-soluble factor is gradually reduced, as extracts from older wounds are used. The extracts from four to five days old wounds do not seem to influence the cell growth, nor do extracts from other older wounds influence the cell growth *in vitro*. In the experiments with the water-soluble factor the extracts from one hour up to one to two days old wounds seem to have a slightly stimulating effect on the growth. The extracts from older wounds, up to 12 to 13 days old, on the other hand, seem to inhibit the growth. Extracts from six to ten days old wounds seem to have the most marked effect in this respect, inhibiting the growth by up to 60 per cent. The growth-inhibiting action is

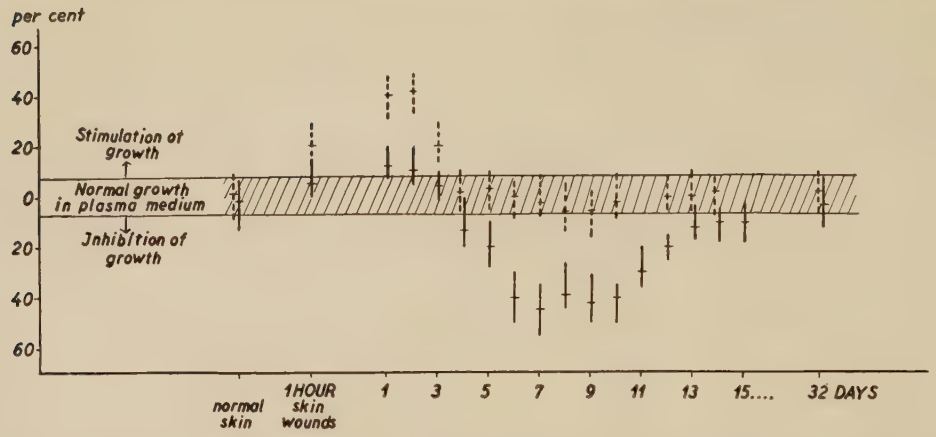


Fig. 4. The figure shows the effect of wound extracts on the growth of fibroblast culture. The broken line indicates the effect of the physiological salt soluble factor. The whole line indicates the effect of the water soluble factor.

not distinctly reduced until extracts from 13 to 15 days old wounds are used.

With regard to the effect on the cell growth, extracts from 32 days old wounds differ from the other extracts in that they have no effect on the growth. The growth of the experimental cultures shows the same variations as that of the control cultures.

Finally, in Fig. 5, the growth inhibition effect of the wound extract is plotted as a function of the dye uptake of the granulation tissue. The last-named is recorded in the form of the dye binding index (D), (see Fig. 2). As seen from the diagram the growth-inhibiting effect is increased when D reaches and exceeds 1.5. There seems to be a distinct correlation between the growth-inhibiting effect of the wound extract and the power of the granulation tissue to take up dye.

It has been shown above that the dye-uptake varies constantly during the healing process. It would therefore be of interest also to study the histological picture. For that purpose the granulation tissue from a series of rats killed according to the following time-schedule was examined: 1, 2, 3, 4, 5, 7, 8, 10, 11, 14, 15 days and 1 month after the wound had been made.

As early as the second day the scars in the preparations fixed with 4 per cent basic leadacetate (6) and stained with toluidine blue showed slight metachromasia which increased gradually, becoming very marked on the seventh day. The metachromasia then seemed to remain the same on the whole, possibly with a slight decrease on the 15th day and disappeared

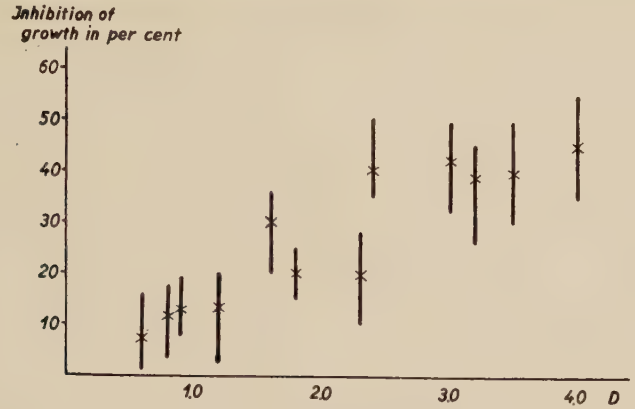


Fig. 5. Inhibition of growth plotted against dye binding index (D).

entirely after a month. The metachromatic changes were observed chiefly in the granulation tissue but also, although less pronounced, in the adjacent tissue.

In the preparations fixed in Carnoy and Zenker and stained with silver (Gomori method) and azan the writers studied the appearance of the fibrillar structures in the granulation tissue. With both stains fine fibrils appeared in the granulation tisssue at a relatively early stage (third-fourth day). The argyrophilic fibrils seemed to appear earlier than those stained with azan. Gradually the fibrils became coarser and arranged themselves side by side in the granulation tissue. Toward the 15th day the collagen fibrils were on the whole as coarse as the fibrils of the normal skin.

With regard to the activity of phosphatase (Gomori method) in the granulation tissue, abundant cells rich in alkaline phosphatase were observed in the tissue closest to the wound edge, only 24 hours after the wound had been made. This picture predominated up to the fourth day, when the number of phosphatase-positive cells began to decrease. Simultaneously a distinct activity of alkaline phosphatase was observed in the cells of the new tissue. The free phosphatase-positive cells disappeared gradually and the phosphatase activity of the granulation tissue increased. Fifteen days after making the wound alkaline phosphatase was still present in the granulation tissue, although a slight decrease was noticed. A one month old scar presented a fully normal picture. The appearance of alkaline phosphatase in collagen fibrils is consistent with Fell's and Danielli's (4) observation on wound healing. With regard to acid phosphatase no distinct changes were observed.

DISCUSSION

As the present work is based on the capacity of the tissue to combine with toluidine blue, it would be of interest to discuss the structures and substances which may play a decisive part in this respect.

If normal skin is stained with toluidine blue used in the manner described above, the cell nuclei in particular but also the rest of the tissue will assume a very faint tint. As in normal skin the value for absorbed dye ranges between 2.0 and 3.0 γ per mg of tissue and as this value is obtained for granulation tissue only after extracting it with water, the high value for young granulation tissue must be due to other factors. As seen from Fig. 1 the capacity of the tissue to take up dyes increases with the age of the wound up to eight days after making the wound, whereafter a decrease follows. Since the histological examination shows that fine fibrils begin to appear on the third to fourth day, as the wound becomes gradually filled with granulation tissue, it may be argued that this very increase of cells would be the cause of the increased capacity of taking up dyestuff. The amount of dyestuff is gradually reduced and the histologic picture shows a progressive differentiation of the granulation tissue. This is particularly evident in the increased formation of fibrils. At this stage most of the fibrils stain deeply with azan and consist of ordinary collagen bundles. The possibility that the granulation tissue, as such, would be responsible for the variations in the uptake of dyes may in our opinion be ruled out, partly on account of what has already been said, partly as the values for uptake of dye obtained after water extraction are the same whether the experiment involves normal skin or granulation tissue (see Fig. 1).

As toluidine blue is a basic dye, it should be taken up by acid negative groups in the tissue structure. The most probable groups are 1) proteins; 2) nucleoproteins; 3) high-molecular polysaccharides (10, 15). The work of Paul, Paul, Taylor and Marsters (16, 17) is of special interest. They have shown that the amount of thiamine in wound healing increases to the 9th–12th day, and that the protein content of the repair tissue gradually increases during wound healing, but is always lower than the protein value in normal skin. They have shown that the water content is much higher in repair tissue than in skin. By the eighteenth day the water content begins to approach the value for skin. It may of course be argued that an increase in nuclei with the appearance of the granulation tissue would lead to an increase in nucleoprotein, which in its turn would cause increased uptake of dye. This seems, however, improbable, as the substance giving rise to the uptake

is readily soluble in distilled water at +4° C, which is probably not the case with the nucleoproteins. On staining with toluidine blue before and after extraction for 24 hours with distilled water at +4° C the staining ability of the nuclei shows no noticeable difference. The result seems to be due to some other substance, and as this substance is readily soluble in distilled water, we seem to be concerned with an extracellular substance. As diffuse extracellular metachromasia may be demonstrated histologically in growing tissue and in granulation tissue, and as this reaction is in all probability to be attributed largely to highmolecular sulphuric acid esters, (Lison (11, 12) and others), we consider it likely that the variation in the capacity of the granulation tissue to combine with toluidine blue is caused by substances belonging to the mucopolysaccharide group. A characteristic feature of these substances is that they are readily soluble in water (14). The general occurrence of substances belonging to this group suggests that they are of great biologic importance. A particularly significant fact is that they occur abundantly in growing tissue (7, 9, 19, 20, 21).

Balazs has shown that a characteristic feature of the acid mucopolysaccharides in general is that they inhibit the growth of fibroblasts *in vitro*. This observation is of interest in connection with the present work. The inhibitory effect obtained on adding water extracts from the granulation tissue to fibroblast cultures is in all probability due to the presence of mucopolysaccharides. It is not possible that the effect is brought about by low-molecular substances, as the extracts were dialyzed previously. Another remarkable fact is that this growth-inhibiting effect was not obtained with extracts from young wounds, when the uptake of dyes is still inconsiderable. At this stage a growth-promoting effect is produced, if physiological saline extract from the granulation tissue is added to the cultures. The reason for this is not quite clear. It seems probable, however, that the tissues fluid which is liberated when the wound is made has a growth promoting effect.

The writers do not wish to discuss whether the mucopolysaccharides in the granulation tissue have a directly inhibiting effect on the cell growth or not. In this connection, it is of interest to point out that the growing cell does not change its character, but that differentiation seems to be possible only when the growth has ceased. It is not maintained that the mucopolysaccharides would be the important factors in this respect, but they are undoubtedly considered to affect the tissue concerned. Meyer (13) suggests that the mucopolysaccharides would be of some importance in the formation of fibrils in the connective tissue. This possibility cannot be excluded. Moreover, maximal uptake of dye in seven to nine days old granulation

tissue was observed in which the formation of fibrils is also particularly marked. After this stage there seems to be no noteworthy formation, the fibrils grow instead coarser and gradually assume the character of collagen.

SUMMARY

1. Granulation tissue (24 hours to 30 days old) from rats was examined with regard to the capacity of the tissue to combine with toluidine blue. It was found that the uptake of dyes increased from 2.0–3.0 γ per mg of tissue on the first day, to 7.0 γ per mg of tissue on the sixth to the ninth day, whereupon it decreased until the value for normal skin was reached on the 15th day.

2. The variations in the capacity of taking up dye are probably to be attributed to extracellular mucopolysaccharides. The possibility that the results are due to other substances is considered.

3. Fibroblast cultures were grown with the addition of water extracts from granulation tissue. An inhibiting effect was demonstrated, which reached a maximum with extracts from six to nine days old granulation tissue. Growth of tissue cultures with physiological salt extract from the granulation tissue showed that the extract from young tissue had a slightly growth-promoting effect.

4. In conclusion the authors have tried to combine the results with the histologic picture of wound healing.

REFERENCES

1. Arcy, L., *Physiol. Rev.*, **16**, 327 (1936).
2. Balazs, A., and Holmgren, Hj., *Nature*, **163**, 488 (1949).
3. Danielli, J. F., Fell, H. B., and Kodicek, E., *Brit. J. exp. Path.*, **26**, 327 (1945).
4. Fell, H. B., and Danielli, J. F., *Ibid.*, **24**, 196 (1943).
5. Fischer, A., *Protoplasma*, **26**, 314 (1936).
6. Holmgren, Hj., *Ztschr. f. wissenschaftl. Mikroskopie und f. mikro. Technik*, **55**, 419 (1938).
7. —— *Anat. Anz.*, **88**, 193 (1939).
8. —— *Ztschr. f. mikr.-anat. Forsch.*, **47**, 489 (1941).
9. Holmgren, Hj., and Rexed, B., *Acta Anat.*, **2**, 287 (1947).
10. Klotz, L., *J. Am. Chem. Soc.*, **68**, 2299 (1946).
11. Lison, L., *Arch. de biol.*, **46**, 599 (1935).
12. —— *Compt. rend. Soc. de biol.*, **118**, 821 (1935).
13. Meyer, K., *Advances in protein chemistry*, **2**, 249 (1945).
14. —— *Physiol. Rev.*, **27**, 335 (1947).
15. Michalies, L., *Symposia of quantitative Biol.*, **12**, 131 (1947).
16. Paul, H. E., Paul, M. F., Taylor, J. D., and Marsters, R. W., *Arch. Biochem.*, **7**, 231 (1945).
17. —— *Ibid.*, **17**, 269 (1948).
18. Sandblom, Ph., *Acta chir. Scand.*, **60**, Suppl., 89 (1944).
19. Sylvén, B., *Klin. Wochenschr.*, **44**, 1545 (1938).
20. —— *Ibid.*, **86**, Suppl. 66 (1941).
21. —— *Acta Radiologica*, Suppl. 59 (1945).

L'INVASION D'UN ORGANISME PAR UN VIRUS HEREDITAIRE

RECHERCHES QUANTITATIVES SUR LA VITESSE D'ACQUISITION DE LA SENSIBILITE AU CO^2 PAR LES DROSOPHILES

NADINE PLUS

Institut de Génétique du Centre national de la recherche scientifique, Paris

Appendice mathématique par PHILIPPE L'HÉRITIER

Institut de Biologie physico-chimique, Paris

Reçu le 10 novembre 1949

INTRODUCTION

La sensibilité au CO^2 chez *Drosophila melanogaster* a été découverte en 1938 par L'Héritier et Teissier et a fait depuis l'objet de nombreux travaux (L'Héritier et Teissier (6); L'Héritier et Sigot (7), L'Héritier et Hugon de Scoeux (8); Goldstein (4)), travaux dont une revue a été faite récemment (L'Héritier (9)). Les mouches dites sensibles sont tuées par un contact, même très court, avec le CO^2, alors que les mouches normales, dites résistantes, peuvent séjourner des heures dans ce même gaz sans en être incommodées d'une manière durable. Cette sensibilité est le seul symptôme de la présence dans l'organisme de la mouche d'un agent cytoplasmique autoreproductible, qui a beaucoup de propriétés communes avec les virus, mais qui a été appelé génoïde parce qu'il est normalement inclus dans les gamètes et est ainsi héréditaire.

On sait, depuis les travaux de L'Héritier et Hugon de Scoeux (8), qu'il est possible de transmettre la sensibilité à des mouches résistantes par injection d'un extrait de mouches sensibles. A la suite de l'injection, les symptômes ne se manifestent qu'après un délai assez long, qui a été appelé temps d'incubation. Quand on injecte des lots de mouches résistantes avec le même extrait diversement dilué, on observe que le temps d'incubation s'allonge à mesure que progresse la dilution.

Il m'a paru intéressant d'étudier la forme précise de cette relation entre temps d'incubation et dose. Elle peut, en effet, apporter des renseignements sur le mécanisme d'envahissement de l'organisme de la Drosophile par le génoïde et peut, d'autre part, fournir une méthode permettant d'apprécier le degré de virulence d'un extrait.

L'idée d'utiliser le temps d'incubation ou le temps de survie pour la mesure de la virulence n'est pas nouvelle et plusieurs auteurs l'ont appliquée à l'étude d'infections diverses. Je ne citerai que les travaux de Ray Bryan et Beard (10) sur le Papillome du Lapin et ceux de Bonet-Maury (2) sur le virus herpétique des Souris.

La plupart de ces travaux ont été revus récemment par Cavalli et Magni (3). Ces auteurs ont montré que le temps d'incubation était relié par une relation linéaire au logarithme de la dose. Ils expliquent ce fait en admettant:

1°) que le parasite se multiplie exponentiellement dans l'organisme de l'hôte,

2°) que les symptômes apparaissent quand le nombre des parasites atteint une valeur fixe.

Si N, en effet, représente le nombre de particules virulentes introduites dans un organisme, T le temps d'incubation ou de survie, g l'intervalle qui s'écoule entre deux divisions du parasite et C le nombre de particules nécessaire à la manifestation recherchée, on a:

$$N 2^{\frac{T}{g}} = C$$

D'où l'on déduit aisément:

$$T = \frac{g}{L 2} (LC - LN)$$

Ipsen (5) s'est servi des temps de survie pour étudier la virulence d'émulsions d'organes de souris infectés par *Rickettsia mooseri*. Il a employé une gamme très étendue de doses et a observé que la loi semi-logarithmique était valable dans un certain domaine de doses moyennes, mais ne s'appliquait plus aux doses extrêmes. Pour les doses faibles, T tend à devenir constant et pour les doses fortes, le paramètre g devient plus grand. Ipsen propose une formule dérivée de la loi semi-logarithmique, mais qui prend en considération l'approche asymptotique de T vers une valeur qui n'est jamais dépassée.

Il importe d'ailleurs de noter que le cas de la sensibilité au CO^2 est assez

différent des infections étudiées par ces auteurs. Ils ont utilisé, en effet, pour leurs recherches sur le temps d'incubation, l'inoculation d'agents pathogènes à des Vertébrés, chez lesquels entrent certainement en jeu des mécanismes de défense de l'organisme. Dans le travail dont les résultats vont être exposés, au contraire, l'agent infectieux n'est apparemment pas pathogène puisqu'il ne trouble nullement l'organisme dans les conditions biologiques normales. L'intervention de phénomènes d'immunité est donc peu probable à priori et, effectivement, n'a jamais été suggérée par aucun des faits recueillis au cours des nombreuses recherches réalisées sur la sensibilité au CO^2.

TECHNIQUE

Les extraits virulents sont préparés à partir d'une souche de drosophiles sensibles, désignée par le symbole *σ–e*, homozygote pour un des allèles du gène ebony. Les mouches résistantes employées comme hôtes sont de génotype sauvage Oregon *R–C*. Dans chaque expérience, les mouches résistantes proviennent d'une même bouteille d'élevage. Elles sont prises 48 heures après l'émergence imaginale et mises sur du milieu nutritif neuf 24 heures avant l'injection. Dans toutes les expériences, les mouches injectées sont donc de même génotype, de même âge et dans le même état physiologique. J'ai vérifié, d'ailleurs, au cours d'essais préalables, que ces facteurs n'exercent qu'une influence secondaire sur le temps d'incubation. Le sexe ne le modifie pas; aucun effort n'a donc été fait pour trier les mâles des femelles dans les lots de drosophiles soumises aux injections.

L'extrait infectieux est obtenu à partir de 300 à 500 mouches sensibles que l'on broie soigneusement dans un petit mortier avec une pincée de sable de Fontainebleau et quelques gouttes de Ringer. La pâte est ensuite amenée à une dilution connue avec du Ringer, puis centrifugée pendant 30 minutes à la vitesse de 3 500 tours minute. Le liquide surnageant peut être à nouveau dilué avant l'injection. Toutes les manipulations: broyat, centrifugation, dilutions, sont faites à 0° pour éviter l'altération du virus.

Chaque dilution de l'extrait infectieux est injectée à une cinquantaine de mouches résistantes. L'injection est faite dans l'abdomen et poussée jusqu'à ce que la trompe se déplie. Dans ces conditions, la quantité de liquide injectée est sensiblement constante et égale à 1/3 de mm^3. Les mouches injectées sont maintenues à 20°, dans des tubes contenant du milieu nutritif, qui sont renouvelés très fréquemment pour assurer aux animaux une nutrition parfaite.

Pour déterminer le moment où apparaissent les symptômes de la sensibilité, les mouches sont plongées tous les jours, pendant 1/4 d'heure, dans une atmosphère de CO^2 pur, à la température de 12°,5. Pour réaliser cela, chaque lot de mouches est introduit dans une petite chambre à gaz; puis l'ensemble des chambres est immergé dans un bain à la température voulue et parcouru par le même courant de CO^2. Les mouches sont ensuite sorties, comptées et introduites dans un tube. Au bout de quelques minutes, les résistantes se réveillent, alors que les sensibles restent paralysées et plus ou moins immobiles. On peut ainsi séparer et compter ces dernières. Les mouches résistantes sont ensuite remises à l'étuve à 20° jusqu'au test du jour suivant. On établit ainsi, pour chaque lot, une sorte de table de mortalité. Les mouches sensibles sont parfois assez difficiles à identifier, car les symptômes de la sensibilité ne sont pas toujours très nets quand ils apparaissent pour la première fois. C'est pourquoi j'ai adopté comme règle de ne compter comme sensibles que les mouches incapables de marcher après le test. Celles qui présentent des symptômes plus légers d'intoxication sont considérées comme résistantes. Il arrive que quelques unes de ces mouches meurent avant le test du jour suivant; elles sont alors ajoutées aux sensibles observées ce jour là. Quand les animaux sont bien nourris, la mortalité naturelle est, en effet, négligeable jusqu'au 25ème jour de la vie imaginale et le temps d'incubation ne dépasse qu'exceptionnellement cette limite.

RÉSULTATS

I°) Comme il a été dit au chapitre technique, les expériences conduisent à des tables de mortalité comprenant au moins 50 mesures individuelles. Ces distributions sont très sensiblement normales. Dans le tableau I, sont présentés, à titre d'exemple, les chiffres obtenus avec un lot de 89 mouches injectées avec un extrait dilué 1.000 fois. Cette expérience est choisie à dessein parmi celles qui ont fourni une des distributions les plus irrégulières.

Malgré cette apparence irrégulière, cette distribution n'est pas significativement différente de la loi normale. Le χ^2 de conformité est en effet égal à 9,54 pour 9 degrés de liberté, soit une probabilité de 50 % environ. L'écart-type σ_t des distributions de temps d'incubation est d'autant plus grand que la moyenne T est plus élevée. J'ai constaté empiriquement que la formule suivante est valable pour l'ensemble des distributions observées:

$$\frac{\sigma_t}{T-8,5} = 0,36$$

8,5 correspondant à peu près au temps d'incubation minimum, on voit donc que le coefficient de variabilité de ce temps est constant dans un lot de mouches, si on le compte à partir de sa valeur minima.

TABLEAU I

Exemple de table de mortalité.

Temps d'incubation	Nombre de mouches
10.5	2
11.5	3
12.5	11
13.5	17
14.5	23
15.5	9
16.5	14
17.5	5
18.5	2
19.5	2
20.5	1
	89

II°) La distribution des temps d'incubation étant normale, il est raisonnable de prendre, comme mesure de l'effet d'une dose, la moyenne arithmétique des observations effectuées sur les différents individus d'un même lot. Pour calculer cette moyenne, on attribue à chaque individu un chiffre correspondant au milieu de l'intervalle entre deux tests successifs. Par exemple, toutes les mouches qui, résistantes à la fin du $8^{ème}$ jour, se sont montrées sensibles à la fin du $9^{ème}$, sont comptées comme ayant présenté un temps d'incubation de 8,5 jours.

Lorsque l'on compare les moyennes ainsi calculées pour une série de dilutions successives d'un même extrait, on observe approximativement la relation linéaire entre temps d'incubation et logarithme de la dose signalée par les auteurs précédemment cités. Sur un graphique où l'on porte en ordonnée ces moyennes et en abscisse le logarithme de la dilution, les points représentatifs sont à peu près en ligne droite.

III°) Chaque extrait ne peut pratiquement fournir que 6 points au maximum. Pour étudier avec plus de soin la relation entre temps d'incubation et dose, il est nécessaire de pouvoir rapprocher les résultats d'expériences différentes. Pour cela, il faut rapporter les abscisses à une origine commune. Remarquons, en effet, que le logarithme de la dilution représente, avec une origine inconnue, variable suivant la virulence de l'extrait qui a été dilué,

le logarithme du nombre de particules infectantes contenues dans le volume d'injection. On peut tenter d'estimer directement ce nombre en appliquant à chaque extrait la méthode classique des dilutions, dont le principe est le suivant. Si on injecte à des mouches un extrait concentré, elles deviennent toutes sensibles; mais, quand on utilise une dilution suffisante, une fraction d'entre elles reste résistante. Dans beaucoup de cas analogues, les auteurs interprètent cette ségrégation comme révélant l'existence d'une variation dans la résistance à l'infection des différents individus. Mais, dans le cas de la sensibilité au CO^2, aucun phénomène de défense de l'organisme de la mouche contre l'invasion du génoïde ne paraît intervenir. On peut alors, raisonnablement, admettre l'hypothèse simple que les mouches restées résistantes représentent celles qui, fortuitement, n'ont point reçu de particules efficaces.[1] Il est normal de considérer que la distribution des nombres de ces particules contenues dans la masse d'injection suit la loi de Poisson. Si l'on admet alors qu'un génoïde est suffisant pour rendre une mouche sensible, la fréquence p des individus restés résistants représente le premier terme de cette loi et la moyenne m du nombre des particules efficaces (unités infectantes) est donc égale à $-Lp$. Cette méthode de mesure du titre des suspensions de génoïdes a été utilisée antérieurement par F. Hugon de Scoeux, qui en a mis au point les détails (communication verbale).

La légitimité de la méthode ressort de l'accord obtenu entre les déterminations correspondant aux diverses dilutions d'un même extrait. Or, en première approximation, cet accord est bien réalisé.

Fréquemment toutefois, on obtient, avec les dilutions fortes, des chiffres anormalement élevés. J'ai étudié systématiquement, à ce point de vue, les données recueillies par F. Hugon de Scoeux et les miennes. J'ai comparé, dans ce but, les déterminations du titre d'un même extrait, faites d'une part avec une dilution forte, d'autre part avec la dilution 10 fois moins forte. Les 8 valeurs disponibles pour le rapport de ces deux estimations admettent comme moyenne 1,17. Cette erreur systématique peut être due à deux facteurs, qui peuvent coexister et dont les actions peuvent s'ajouter.

1°) Une aggrégation des particules dans les extraits concentrés, les amas se désagrégeant lorsqu'augmente la dilution.

2°) L'erreur sur le volume injecté. Celle-ci peut provenir soit de l'imprécision dans l'injection elle-même, soit du rejet éventuel par les mouches d'une partie du liquide. Comme il est montré dans l'appendice, cette erreur conduit à une estimation du titre plus élevée pour les dilutions fortes que pour les dilutions basses.

[1] J'ai d'ailleurs vérifié que l'extrait de ces mouches n'est pas virulent.

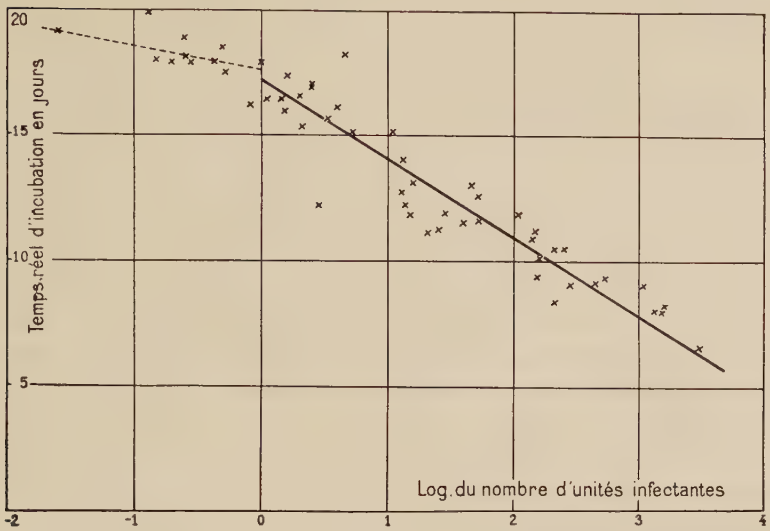


Fig. 1. Variation du temps moyen d'incubation en fonction du logarithme du nombre d'unités infectantes, calculé d'après le pourcentage de mouches résistantes observées aux fortes dilutions.

Quelle que soit son origine, l'erreur systématique qui vient d'être signalée est petite et n'introduit pas de gros écarts dans la mesure du titre. Lorsque les résultats de plusieurs dilutions sont disponibles, j'adopte pour celui-ci la moyenne pondérée des différentes déterminations. Celles-ci sont affectées d'un poids égal à l'inverse de leur variance statistique. La variance de l'estimée du nombre d'unités infectantes dans l'extrait initial, non dilué, est $\frac{d^2}{p}\frac{1-p}{n}$, p étant le pourcentage de mouches résistantes, calculé sur n individus injectés avec cet extrait dilué d fois. Cette expression augmente beaucoup avec d, malgré l'augmentation de p. Il en résulte que l'emploi de la moyenne pondérée conduit à accorder une faible importance aux chiffres obtenus avec des dilutions fortes.

Il faut remarquer que le titre, déterminé par cette méthode, est mesuré en unités infectantes et qu'une unité ne correspond pas nécessairement à une particule unique. La cohérence des résultats obtenus à partir des différentes dilutions d'un même extrait permet, en effet, d'affirmer que les particules sont indépendantes dans leur efficacité, c'est à dire dans leur probabilité d'implantation dans l'organisme, mais ne fournit aucune indication sur la valeur de cette probabilité qui reste une inconnue. Il se pourrait qu'elle ait une valeur très différente de I, qu'une seule particule sur 10 ou

sur 100, par exemple, parvienne à s'implanter. L'unité infectante équivaut alors à 10 ou 100 particules introduites.

La méthode des dilutions permet donc de comparer directement les résultats d'expériences différentes et d'étudier la relation qui lie le temps d'incubation au nombre d'unités infectantes.

Quatorze expériences indépendantes ont été réalisées, comportant chacune un certain nombre de dilutions d'un même extrait. En tout, 53 déterminations du temps d'incubation ont été obtenues, représentant chacune la moyenne d'au moins 50 drosophiles. Les résultats sont présentés sur la figure 1, dans laquelle l'abscisse correspond au logarithme du nombre d'unités infectantes contenues dans la masse d'injection, nombre calculé d'après la ségrégation observée aux hautes dilutions. De l'examen de la figure se dégagent les faits suivants.

a) Les points représentatifs sont relativement dispersés autour d'une loi moyenne.

b) En première approximation, le temps d'incubation est effectivement, comme dans la théorie classique, relié linéairement au logarithme du nombre d'unités infectantes.

c) Cette linéarité n'est cependant bien réalisée que dans la région moyenne des abscisses. Dans la partie gauche, à partir de l'abscisse 0 environ, le temps d'incubation paraît rester sensiblement constant.

d) Dans la région des fortes concentrations, l'accroissement du temps d'incubation pour un même écart d'abscisse semble devenir plus faible.

DISCUSSION DES RÉSULTATS

I°) Comme il est remarqué plus haut, la relation semi-logarithmique entre temps d'incubation et dose est couramment interprétée par le mécanisme suivant.

L'agent infectieux, introduit dans l'organisme, s'y multiplie exponentiellement et le symptôme se manifeste quand un niveau déterminé est atteint par cette multiplication. Si on adopte cette conception, la pente de la droite permet alors de calculer le taux d'accroissement des particules infectieuses. Cette pente, estimée par la méthode d'ajustement décrite ci-dessous est de –3,12, ce qui signifierait qu'il faudrait 3,12 jours pour décupler le nombre de particules contenues dans la mouche. Ce nombre doublerait donc toutes les 22,6 heures. D'autre part, le temps d'incubation maximum observé est de 18,2 jours en moyenne. Il en résulterait que le

seuil à partir duquel la mouche manifesterait les symptômes de la sensibilité serait de 67.000 génoïdes.

Il faut remarquer, toutefois, que dans le cas d'un virus cette conception d'une multiplication exponentielle au sein d'un milieu homogène est probablement trop simple. La multiplication des virus a lieu à l'intérieur des cellules et rien n'est connu actuellement sur la loi formelle à laquelle elle obéit. On peut dès lors, semble-t-il, considérer deux phénomènes dans l'envahissement d'un organisme par un virus, d'une part la multiplication intracellulaire, d'autre part la contamination des cellules les unes par les autres. La vitesse avec laquelle l'organisme manifestera les symptômes de l'infection va dépendre des rythmes de ces deux phénomènes et de leur interaction. Dans quelle mesure un tel mécanisme est-il compatible avec la relation semi-logarithmique observée entre le temps d'incubation et la dose injectée? Dans l'appendice, P. L'Héritier donne une représentation mathématique de la marche du phénomène en partant d'hypothèses simplifiées. Il suppose, en effet, que les génoïdes qui se sont multipliés dans les cellules en diffusent à un rythme constant et seulement lorsqu'une concentration déterminée est atteinte. On peut voir que ce mécanisme d'envahissement d'un organisme aboutit au même résultat global que la multiplication exponentielle dans un milieu continu, mais que la pente de la droite ne correspond plus directement au taux d'accroissement des particules infectantes, elle est une fonction des deux paramètres: temps de saturation d'une cellule et rythme d'émission.

II°) La figure 1 comporte 11 points d'abscisse inférieure à 0 correspondant à de fortes dilutions. Pour voir si dans cette zône le temps d'incubation varie réellement en fonction de la dose, j'ai calculé les éléments de la droite de régression temps sur dose (droite en pointillé de la figure 1). Sa pente est égale à $0{,}41 \pm 1{,}95$, c'est-à-dire qu'elle n'est pas significativement différente de 0. Elle est d'autre part très différente de la pente de la régression dans les régions d'abscisse supérieure à 0. Le temps d'incubation est donc sensiblement constant dès que le nombre moyen de particules par mouche est inférieur à 1. Ce temps maximum est égal à $18{,}2 \pm 0{,}3$ jours. Remarquons que cette indépendance du temps d'incubation vis à vis de la dose est une conséquence nécessaire de l'hypothèse faite pour expliquer l'apparition de mouches résistantes aux dilutions fortes. En effet, la plus grande partie des mouches qui deviennent sensibles ne reçoit alors qu'un seul génoïde efficace, le seul effet de la dilution étant d'augmenter la proportion des résistantes. La période de 18,2 jours correspond donc au temps d'acquisition de la sensibilité par une drosophile à partir d'une particule unique.

Les valeurs obtenues pour le temps d'incubation correspondant à des nombres moyens d'unités infectantes inférieurs à 1, constituent donc une classe à part et ne peuvent pas être étudiés en même temps que les autres. Si on les met de côté, les résultats comportent 42 points d'abscisse supérieure à 0,15, correspondant donc à des expériences dans lesquelles la masse d'injection contenait au moins 1,4 génoïde efficace en moyenne. L'estimation à partir de ces points des paramètres de la relation entre les deux grandeurs ne peut se faire par la méthode classique de la régression. Remarquons, en effet, que dans ce cas l'abscisse et l'ordonnée sont toutes les deux sujettes à des erreurs aléatoires. Le nombre de particules infectieuses caractéristique d'une injection ne peut être fixé à priori et n'est calculé qu'à partir du nombre de mouches résistantes observées dans un échantillon. Différents auteurs ont étudié le problème statistique de la détermination de la ligne idéale lorsque les deux variables sont ainsi aléatoires. La plupart de ces travaux ont été revus par Bartlett (1). J'ai adopté la méthode proposée par G. Teissier (11). Elle consiste à choisir la droite d'équation:

$$Y - \bar{y} = \frac{\sigma_y}{\sigma_x}(X - \bar{x})$$

dans laquelle $\bar{x}$ et $\bar{y}$ désignent les moyennes des deux variables, σ_x et σ_y les écarts types de leur distribution. Cette droite est représentée en trait plein sur la figure 1. Son équation est Y–12,30 = –3,12 (X–1,56).

On voit qu'en première approximation elle représente assez bien la loi moyenne qui relie les deux grandeurs. On peut cependant noter qu'aux deux extrémités la plupart des points se situent au dessus de la droite, alors qu'ils sont en dessous dans la région moyenne. La pente paraît donc être plus grande dans la zône des doses élevées qu'à l'autre extrémité du domaine exploré. Cette constatation m'a conduit à analyser séparément les données relatives à ces deux régions. La pente de la droite, ajustée par la méthode décrite ci-dessus aux points d'abscisse comprise entre 0,15 et 1,80, est $4.28 \pm 0{,}49$; celle qui correspond aux abscisses supérieures à 1,80 est par contre $2{,}94 \pm 0{,}40$. La différence $1{,}34 \pm 0{,}63$ est juste significative au niveau de probabilité 5 %. Le même fait ressort plus nettement encore de la comparaison suivante. Dans 13 des expériences, l'extrait de mouches sensibles a été dilué successivement au dixième, au centième et au millième. On peut calculer, pour chacune d'elles, l'écart des temps d'incubation correspondant aux dilutions 10 et 100 d'une part, 100 et 1.000 de l'autre. Les chiffres sont donnés dans le tableau II. On voit que 12 fois sur 13 l'écart est nettement plus grand dans le second cas que dans le premier.

Il semble donc certain que la relation entre le temps d'incubation et le logarithme de la dose n'est pas strictement linéaire, mais que sa pente tend à diminuer dans la région des hautes doses. Malheureusement aucun des extraits préparés par la méthode décrite ci-dessus n'a fourni de concentrations très élevées en unités infectantes et aucun point de la figure 1 n'a d'abscisse supérieure à 3,50. Des méthodes sont actuellement à l'étude qui permettront sans doute d'obtenir des suspensions plus virulentes. Il me paraît donc prématuré d'essayer d'extrapoler à partir des résultats actuels et de préjuger de la forme globale de la relation entre temps d'incubation et dose. A fortiori, est-il trop tôt pour donner une interprétation biologique au changement de pente observé.

Tableau II

Ecarts entre les temps d'incubation correspondant, pour une même expérience, aux dilutions 10 et 100 d'une part, 100 et 1.000 de l'autre.

Dilutions 10 et 100	Dilutions 100 et 1.000
2.2	3.5
3.8	5.2
1.8	3.0
0.8	5.6
1.4	2.4
2.4	4.1
2.7	4.2
2.8	1.3
1.3	3.3
2.8	3.2
2.7	4.2
2.7	3.4
2.3	2.9

UTILISATION DES RÉSULTATS POUR LA DÉTERMINATION DU TITRE D'UN EXTRAIT VIRULENT

Dès maintenant, en tous cas, la relation mise en évidence entre le temps d'incubation et le nombre d'unités infectantes introduites dans une mouche, permet de déterminer le titre d'un extrait, même en l'absence d'apparition de mouches résistantes. Cette méthode présente sur la méthode des dilutions exposée ci-dessus les avantages suivants. D'une part, elle fournit le

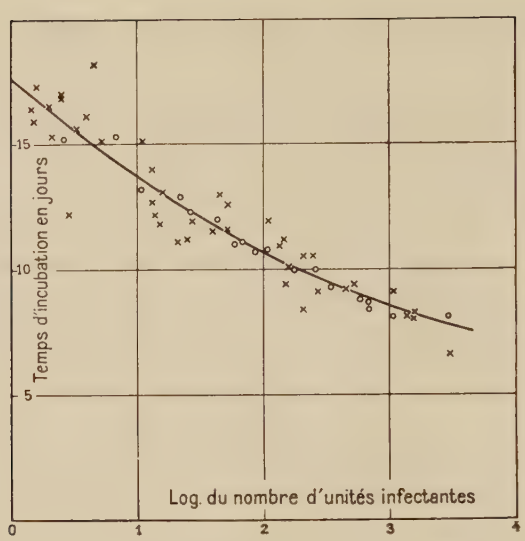


Fig. 2. Courbe du deuxième degré ajustée aux données de la fig. 1, représentées par des x. ○ abscisses déterminées à partir de cette courbe, en faisant la moyenne des résultats correspondant aux dilutions d'un même extrait.

résultat plus rapidement et, d'autre part, elle ne nécessite pas une connaissance préalable grossière du titre.

Pour utiliser pratiquement la méthode, il est nécessaire de disposer d'une bonne estimation empirique de la loi moyenne entre les deux grandeurs dans le domaine étudié. Or on a vu que cette loi n'est qu'approximativement linéaire. L'utilisation de la droite de la figure I conduirait donc à surestimer le titre de part et d'autre de la zône des concentrations moyennes. Il est possible de tenir compte de cette courbure en ajustant aux données une loi du second degré. La courbe correspondante d'équation $Y = 0{,}45\ x^2 - 4{,}39\ x + 17{,}65$ est représentée sur la figure 2.

Elle peut être utilisée comme courbe d'étalonnage pour estimer le nombre d'unités infectantes contenues dans une suspension virulente. En principe, une seule dilution peut suffire. Cependant, pour la précision du résultat, il est nécessaire que le point tombe dans la région des concentrations moyennes. S'il est trop à gauche, l'existence d'un palier rend inutilisable le temps d'incubation comme mesure de la dose. S'il est trop à droite, le fait que dans cette région le temps varie plus lentement en fonction de la dose rend la mesure imprécise. Il est donc généralement préférable d'injecter en même temps, à des lots de drosophiles séparés, plusieurs dilutions de l'extrait. Chacune fournit, par lecture directe sur la courbe, une estimation du loga-

rithme du nombre d'unités infectantes contenues dans la masse d'injection. La même quantité pour l'extrait initial non dilué est obtenue en ajoutant à cette lecture le logarithme de la dilution. Le titre est alors calculé à partir de la moyenne de ces logarithmes.

Cette méthode a permis de représenter sur la figure 2 les résultats de 5 expériences comportant chacune les déterminations des temps d'incubation pour au moins 3 dilutions différentes d'une suspension virulente. Aucune de ces dilutions n'était suffisamment élevée pour donner lieu à une ségrégation entre individus qui deviennent sensibles et individus qui restent résistants. Les 18 points obtenus sont les cercles de la figure 2. On voit que les écarts par rapport à la courbe ajustée aux données précédentes sont faibles; ils correspondent cependant à 13 degrés de liberté, un seul ayant été pris, dans chaque expérience, pour la détermination du titre de l'extrait.

La méthode qui vient d'être décrite est actuellement utilisée pour différentes recherches poursuivies sur la sensibilité au gaz carbonique chez la Drosophile. En particulier, elle permet de déterminer facilement la diminution du titre des suspensions de génoïdes après irradiation par les rayons *X* ou les rayons ultra-violets.

RÉSUMÉ

Le temps mis par des drosophiles pour devenir sensibles (temps d'incubation), après l'injection d'une suspension virulente de génoïdes, a été mesuré et mis en relation avec le nombre des unités infectantes introduites. Celui-ci est déterminé à partir du pourcentage de mouches restées résistantes après l'injection d'une suspension suffisamment diluée.

Dans la région des concentrations moyennes, le temps d'incubation est linéairement proportionnel au logarithme du nombre de particules. Quand la concentration moyenne descend au dessous d'une unité par mouche, le temps reste fixé à une valeur maximum. Pour les concentrations élevées, la relation n'est plus linéaire, sa pente tendant à diminuer.

Une méthode pour la détermination du titre d'un extrait est dérivée de la relation trouvée.

SUMMARY

The time required for drosophilas to become CO^2 sensitive (incubation time) following injection with a suspension of genoïdes, has been measured and correlated with the number of infectious units introduced into the fly.

This number is derived from the percentage of flies which remain resistant following injection with a sufficiently diluted suspension.

With moderately concentrated suspensions, a linear relation is observed between incubation time and the logarithm of infectious units. However, when the mean concentration falls to less than one unit per injected fly, the incubation time remains at a fixed maximum value. For high concentrations of genoïdes, the semilogarithmic linear relation does not hold, its slope becoming less steep.

A new method for assaying the titer of a suspension of genoïdes is derived from the established relation.

LITTÉRATURE CITÉE

1. Bartlett, M., *Biometrics*, **4**, 14 (1949).
2. Bonet-Maury, P., *C. R. Soc. Biol.*, **135**, 385 (1941).
3. Cavalli, L., and Magni, G., *Heredity*, **1**, 127 (1947).
4. Goldstein, L., *Bul. biol. Fr. et. Belg.*, **83**, 177 (1949).
5. Ipsen, J., *Schw. Zeit. f. Path. Bakt.*, **7**, 129 (1945).
6. L'Héritier, Ph., et Teissier, G., *Pub. Lab. E. N. S.*, Paris, *Biologie, fasc.* **1** (1945).
7. L'Héritier, Ph., et Sigot, A., *Bul. biol. Fr. Bel.*, **80**, 171 (1946).
8. L'Héritier, Ph., et Hugon de Scoeux, F., *Bul. biol. Fr. Bel.*, **81**, 70 (1947).
9. L'Héritier, Ph., *Heredity*, **2**, 325 (1948).
10. Ray Bryan, W., and Beard, J. W., *J. inf. dis.*, **65**, 306 (1939).
11. Teissier, G., *Biometrics*, **4**, 14 (1948).

APPENDICE MATHÉMATIQUE

par PHILIPPE L'HÉRITIER

1. *Marche du processus d'envahissement d'un organisme par un agent infectieux à multiplication endocellulaire*

Le processus comprend deux aspects: l'un est la multiplication de l'agent à l'intérieur des récepteurs cellulaires infectés, l'autre sa diffusion vers de nouveaux récepteurs. On peut supposer, pour simplifier le problème, que ces deux phénomènes correspondent à deux phases distinctes, séparées dans le temps. Un récepteur infecté ne diffuse de particules susceptibles de contaminer les éléments voisins, que lorsque la multiplication interne de l'agent y a atteint un certain seuil limite. La marche globale du phénomène est alors indépendante des modalités particulières de cette multiplication et ne dépend que de deux paramètres: le temps t_r nécessaire pour la

saturation d'un récepteur et le rythme r avec lequel un récepteur rempli émet des particules contaminatrices. Le calcul ci-dessous est basé sur l'hypothèse simple que ces deux paramètres ont une valeur uniforme pour tous les récepteurs et pendant toute la marche de l'infection.

Soit alors y_t le nombre de récepteurs saturés au temps t. L'accroissement dy représente le nombre de récepteurs nouveaux contaminés pendant l'intervalle de temps dt au temps $t–t_r$. Or, à cette époque, le nombre des récepteurs saturés était y_{t-tr} et le rythme total de diffusion des particules $r \cdot y_{t-tr}$. En admettant que toute particule réussit à contaminer un récepteur nouveau différent, on aboutit donc à l'équation

$$dy = r y_{t-tr}\, dt$$

Il est facile de voir qu'une solution est:

$$y = e^{\lambda t}$$

λ ayant dans cette formule la valeur racine de l'équation:

$$\lambda = r e^{-\lambda tr}$$

Dans les hypothèses considérées, le nombre des récepteurs saturés croît donc exponentiellement avec le temps.

Toutes les hypothèses formulées ci-dessus supposent que le nombre total des récepteurs de l'organisme est suffisamment grand pour que toute particule virulente diffusée dispose, pendant toute la durée du processus d'installation de la sensibilité au CO^2, d'une chance constante de rencontrer un récepteur vierge. Il peut évidemment, à priori, ne pas en être toujours ainsi. Pendant la dernière phase de l'envahissement de l'organisme par l'agent virulent, les récepteurs libres sont certainement raréfiés et la probabilité pour qu'une particule déterminée parvienne à en contaminer un est plus faible qu'au début du processus. Il se peut que cette phase soit atteinte avant l'apparition des symptômes de la sensibilité.

Plusieurs hypothèses sont possibles sur le mécanisme intime du phénomène et, en l'absence de données biologiques, il m'a paru sans grand intérêt d'envisager les lois formelles auxquelles elles conduisent pour l'augmentation du nombre des récepteurs saturés. Par analogie avec ce qui s'observe dans le cas de la multiplication de microorganismes dans un milieu limité, on peut prévoir, en tous cas, que le taux d'accroissement dy/y ne reste pas constant, mais décroît lorsqu'on s'approche de l'état de saturation totale de l'organisme, qui n'est atteint qu'asymptotiquement.

2. *Influence de l'erreur sur le volume d'injection dans le calcul du titre d'une suspension virulente par la méthode des dilutions*

Appelons $\bar{v}$ le volume moyen injecté à chaque individu et N le titre, c'est à dire le nombre de particules contenues dans un volume $\bar{v}$ de l'extrait virulent initial. Nous supposerons que le volume réel injecté v est distribué normalement autour de $\bar{v}$ avec un écart type σ_v. Si on utilise une dilution l, la probabilité liée à v que l'injection n'apporte pas de particules, donc que la mouche reste résistante, est:

$$e^{-\frac{Nv}{l\bar{v}}}$$

La probabilité totale du même évènement, étendue à toutes les valeurs de v, est:

$$p = \int_{v_1}^{v_2} \frac{dv}{\sigma_v \sqrt{2\pi}} e^{-\frac{1}{2}\frac{(v-\bar{v})^2}{\sigma_v^2}} e^{-\frac{Nv}{l\bar{v}}}$$

v_1 et v_2 étant les limites de la variation de v, pratiquement $\bar{v} \pm 3\,\sigma_v$. Cette formule peut, par une transformation algébrique simple, se mettre sous la forme:

$$p = \int_{v_1}^{v_2} \frac{dv}{\sigma_v \sqrt{2\pi}} e^{-\frac{1}{2\sigma_v^2}\left[\left(v-\bar{v}+\frac{N\sigma_v^2}{l\bar{v}}\right)^2 + \frac{2N\sigma_v^2}{l} - \frac{N^2\sigma_v^4}{l^2\bar{v}}\right]}$$

ou encore: $$p = e^{-\frac{N}{l}\left(1-\frac{N\sigma_v^2}{2l\bar{v}^2}\right)} \int_{v^1}^{v^2} \frac{dv}{\sigma_v \sqrt{2\pi}} e^{-\frac{1}{2\sigma_v^2}\left(v-\bar{v}+\frac{N\sigma_v^2}{l\bar{v}}\right)^2}$$

La valeur de l'intégrale serait manifestement l'unité, si la variation de v pouvait, comme dans la distribution normale théorique, s'étendre de $-\infty$ à $+\infty$. Elle en est de toute façon très proche, puisque l'expression $\frac{N\sigma_v^2}{l\bar{v}}$ n'est qu'une fraction de σ_v.
On a donc en définitive:

$$p = e^{-\frac{N}{l}\left(1-\frac{N\sigma_v^2}{2l\bar{v}^2}\right)}$$

L'estimation N' du titre, faite à partir de la fréquence des résistantes, ob-

servée après l'injection d'une dilution l, est donc reliée à la vraie valeur N par la relation:

$$N' = N\left(1 - \frac{1}{2}\frac{N\sigma_v^2}{l\bar{v}^2}\right)$$

Le titre trouvé est donc toujours plus faible que le titre réel, l'erreur absolue étant proportionnelle au carré du titre et au carré du coefficient de variabilité du volume d'injection. Lorsque le titre d'un même extrait est déterminé à l'aide de différentes dilutions, l'erreur, plus faible aux dilutions élevées, conduit donc à une valeur plus forte dans ce dernier cas.

Il est difficile d'avoir une idée sur la valeur réelle du coefficient $\sigma_{v/\bar{v}}$. Indépendamment des variations assez faibles du volume de liquide introduit dans chaque mouche, deux phénomènes peuvent contribuer à le grossir. D'une part, certaines mouches rejettent une partie du liquide, d'autre part, celui-ci n'est pas toujours introduit dans la même région de l'abdomen et il se peut que la probabilité d'implantation d'une particule virulente varie suivant sa localisation particulière. Cette variation agira formellement comme une fluctuation sur les volumes injectés.

Pour obtenir une limite supérieure de l'importance de cette erreur on peut donner au coefficient de variabilité $\frac{\sigma_v}{\bar{v}}$ la valeur 0,5, ce qui est sûrement trop fort. Si on mesure alors le titre d'un même extrait, d'une part, avec une dilution contenant en moyenne I unité infectante par volume d'injection, d'autre part avec une dilution 10 fois plus forte, n'en contenant plus que $\frac{1}{10}$, le rapport des 2 valeurs obtenues est:

$$\frac{1 - \frac{1}{20}(0{,}5)^2}{1 - \frac{1}{2}(0{,}5)^2} = 1{,}14$$

Ce nombre est très voisin du chiffre moyen trouvé en faisant le rapport des déterminations expérimentales du titre d'un même extrait obtenues avec les mêmes dilutions. L'erreur sur les volumes doit donc être un élément important de l'écart systématique trouvé entre celles-ci. Cependant comme la valeur assignée au coefficient de variabilité $\frac{\sigma_v}{\bar{v}}$ est sûrement trop forte, l'intervention d'autres facteurs est probable.

AN INVESTIGATION OF THE PHYSICAL CHEMISTRY OF THE CONTRACTILE PROTEINS

OLLE SNELLMAN and BERTIL GELOTTE

Institutes of Biochemistry and Physical Chemistry, University of Uppsala, Sweden

Received November 30, 1949

ENGELHARDT's (8) discovery that the myosin extractable from muscle is a phosphatase which splits a phosphoric acid group from adenosintriphosphate (ATP) opened a new era in muscle research. Later Needham et al. (14) showed that the streaming birefringence and viscosity of solutions of myosin are altered when ATP is added while the original viscosity and birefringence of these solutions are restored when the ATP is broken down by the myosin. This suggested that the protein present in these solutions was involved in the process of contraction. Almost simultaneously with these investigations, Szent-Györgyi and his students began their epoch-making work in muscle biochemistry. They showed that the reaction between ATP and myosin was actually the bridge between the mechanical and chemical processes.

Szent-Györgyi et al. (23) readily established that the muscle protein which had been previously extracted and which was known as myosin was composed of two proteins. These were denominated crystalline myosin (Szent-Györgyi) and actin (Straub), and the complex was denominated actomyosin. The last named was the main component in the earlier myosin solutions, and is now regarded as the contractile protein. Szent-Györgyi's nomenclature is used below.

The principal object of the investigations presented here was physico-chemical characterization of the different protein components.

Initially an investigation was made with the ultracentrifuge and solutions prepared according to Greenstein and Edsall (21). As was to be expected, these solutions contained a number of protein components in varying proportions. In the sedimentation diagrams it was usually possible to distinguish one or two components which could be shown by the reaction with ATP to be actomyosin, one or often two components with similar sedimentation constants which must have been myosin, and finally an unidentified material

Fig. 1. Electron micrograph of an intact cross-striated muscle fibril.

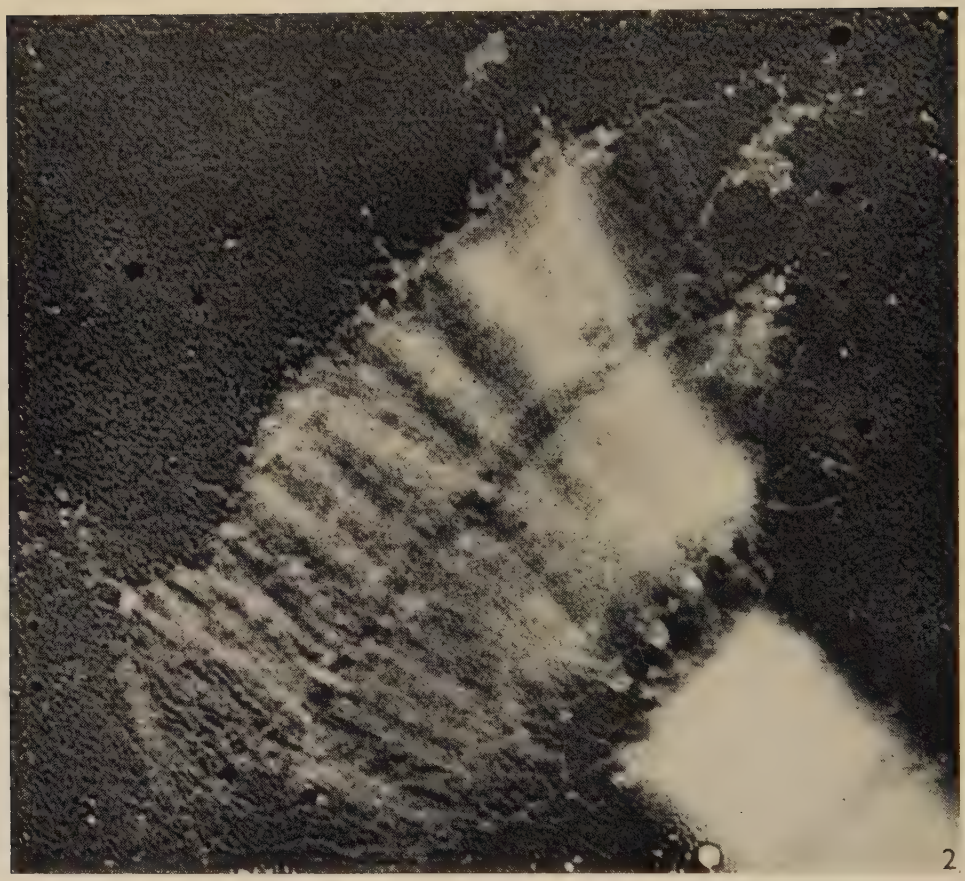


Fig. 2. Electron micrograph of an extracted muscle fibril. The myosin was extracted by brief extraction with 0.3 M KCl.

Fig. 3. Electron micrograph of an extracted muscle fibril.

with a lower sedimentation constant which was sometimes present. The last-named component is now regarded as a fragment of myosin. Myosin can appear as one or two components, depending on how much material is included in the fragment. Since it was this type of solution which was studied before Szent-Györgyi's work became known, it was of interest to show that these solutions did not consist of one single material and that quantitative measurements could not be performed without very exact characterization by some physico-chemical method.

CRYSTALLISZED MYOSIN

Szent-Györgyi (24) isolated a component of the contractile protein myosin by extracting minced rabbit muscle for a short time with 0.3 M KCl. Brief extraction of this kind releases principally myosin and only small quantities of actomyosin. According to Szent-Györgyi the best yield of crystalline myosin, which precipitates when the solution is diluted, is obtained if the muscle is removed directly after the animal's death, immediately cooled with ice water, minced and extracted about 10 minutes at 0° C. The amount of myosin obtained in this way is only a small fraction of the total myosin content in the muscle (10–20 per cent). In order to free the myosin from the accompanying actomyosin, a recrystallization can be performed at a basic pH where the actin will be inactive and remain in solution. Szent-Györgyi calls the latter process a second recrystallization. The amount of actin present as impurity can be tested by viscosimetric examination. If actin is present, the viscosity decreases when ATP is added. With absolutely pure myosin a definite viscosity increase is often observed when ATP is added.

Electron microscope investigations of the crystals (17) show that these consist of long threads packed together. The threads can be partially disaggregated by ultrasonic treatment of crystals. The crystallized myosin is soluble in salt-free water but precipitates to a high degree when salt is added. Minimum solubility is reached at 0.025 M KCl after which the myosin gradually dissolves, becoming completely dissolved at 0.5 M KCl. A stronger streaming birefringence indicates that myosin is still aggregated in solutions up to 0.3 M KCl. Electron microscope photographs (18) of myosin in pure water show that the aggregates consist of long threads. Isolated myosin molecules have not been observed. The investigation indicated that free molecules are readily denatured on the specimen screen.

Since it was of particular interest to note the appearance of the cross striated fibrils subsequent to myosin extraction, a fibril suspension was prepared by breaking up bits of muscle. For this purpose the muscle was subject to the following procedure. The pieces were crushed in a mortar while isotonic buffer at 0° C was added for about 5 min. The product was then suspended in a considerable quantity of cold buffer. The undispersed pieces were removed by brief centrifugation. Then the fibrils were separated by longer centrifugation, suspended in water and studied in an electron microscope. Myosin was extracted from some of the fibrils in the usual way. Since the substance which had held the material together had been lost, the extracted fibrils were flattened out. It seemed as if the extracted myosin were derived principally from the isotropic band, which disappeared (Figs. 1, 2, 3).

The sedimentation diagram in the ultracentrifuge (17) indicated that there was one component, except at low myosin concentrations, where an additional component with a somewhat higher sedimentation constant gradually appeared. This was especially the case with the myosin which was recrystallized twice. In the light of our present knowledge this suggests that something splits off from the myosin so that either shape or hydration changes, and that the altered part of the myosin varies in sedimentation rate.

The diffusion curves were skewed and the diffusion coefficients concentration-dependent. The calculated molecular weight was 1.5×10^6.

This myosin appears as a single component in electrophoresis. Finally, salting-out curves were studied (25) according to Derrien's method (7). A Beckmann spectro-photometer was used to analyze the solutions. The precipitation analysis was performed initially at 20° C. The first experiments gave variable results and were not reproducible. It was evident that it would be extremely difficult to characterize myosin in this way. However, a change was made to measurement of the progress of precipitation at 0°, and reproducible results were obtained (Fig. 4). The myosin precipitated with one principal component and a number of small components. The myosin obtained by fractionation at 0° was pure to a high degree. It was contaminated by only one small component which precipitated at a little lower salt concentration (Fig. 5). When the precipitation curves of the fractionated myosin were studied at 24° C a new component appeared (Fig. 6). An examination of the supernatant salt solution showed that a nucleotide soluble at the salt concentrations employed was partly split off from the myosin.

This investigation shows that myosin can be considered as a material which

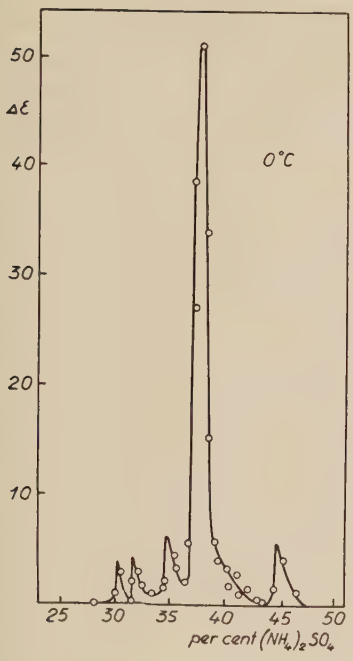


Fig. 4.

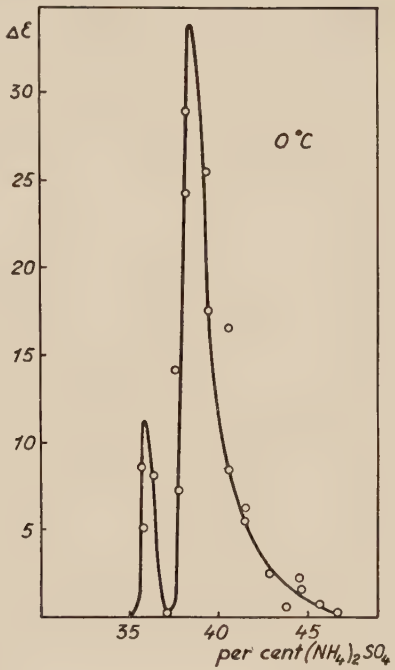


Fig. 5.

Fig. 4. Diagram showing differential salting out of crystallized myosin at 0° C.
Fig. 5. Diagram showing differential salting out of once-fractionated mysoin at 0° C.

is at least within reasonable limits fairly definitely characterized from a physico-chemical point of view. The investigation also shows that it is labile.

A further question is whether myosin from different organs has the same structure. In addition to the myosin described above, which was derived from ordinary cross-striated muscle, heart and uterus myosin were studied. Myosin from lamb heart (11) actually showed certain differences from that described here. It proved impossible to prepare crystallized myosin according to Szent-Györgyi's method; the myosin obtained was insoluble in water as well as in weak salt solutions. It was also possible to obtain heart myosin completely free of actin simply by means of reprecipitation. A different type of concentration dependence was noted in the investigations of the sedimentation constant. In once-recrystallized myosin as well as in Edsall's solutions, the sedimentation constant was a linear function of the concentration, as is often the case with proteins. Regarding heart myosin

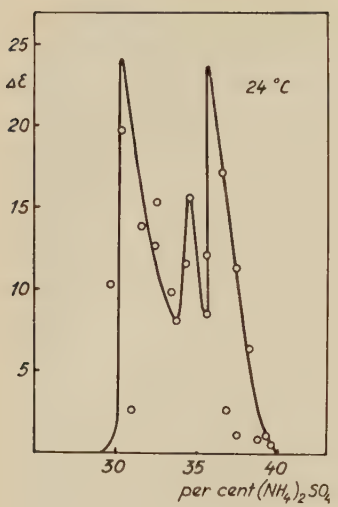


Fig. 6.

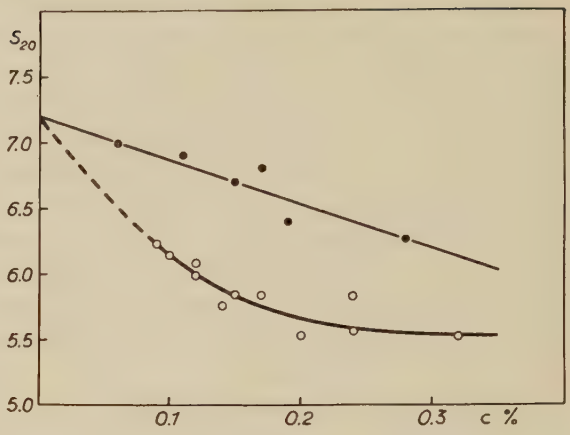


Fig. 7.

Fig. 6. Diagram showing differential salting out of once-fractionated myosin at 24° C.
Fig. 7. Variation of sedimentation constant with concentration. Full circles: once-crystallized myosin. Open circles: heart myosin.

there was, however, a larger dependence, similar to that for fiber molecules (Fig. 7). The latter type was also observed with twice-crystallized myosin. Thus myosin apparently changes structure when completely free of actin. An attempt to prepare crystalline uterus myosin (6) was unsuccessful. The myosin obtained was easily soluble and the preparations were not free of actin and showed greater inhomogeneity, presumably because of the bound actin.

The preliminary conclusion from these investigations is that the firmness with which actin is bound to myosin is greatest in uterus myosin, less in cross-striated myosin and least in heart myosin; in other words it decreases with the increasing physiological activity of the muscle.

Szent-Györgyi and collaborators (3) concluded from enzyme studies that myosin must be built up of a framework substance carrying an indefinite number of substances with different functions. These substances, which Szent-Györgyi calls protins can be split off from the myosin. It appears that none of these is prepared in pure form.

The investigations also confirmed the theory that myosin may break down. An investigation (17) shows that in the course of prolonged treatment with urea, myosin to a large extent breaks down into small components. Brief

treatment does not produce any components of lower sedimentation constant which are apparent in the ultracentrifuge. Nevertheless the myosin changes and does not react with actin in a normal way. The sedimentation constant is substantially higher ($S_0 = 16$). Simultaneously the diffusion constant changes so that the molecular wight is the same as that of untreated myosin. The frictional coefficient diminishes. One possibility is that the extended form has changed.

Another preliminary result, which indicates that myosin can break down completely was obtained by investigating uterus actomyosin. By repeated precipitation an actomyosin was obtained which on ultracentrifugal analysis appeared to contain only a rather small quantity of myosin. When ATP was added to this actomyosin in 0.5 M KCl, an actin component and a quantity of slowly sedimenting material were obtained rather than a myosin component and an actin component as expected. It was clear that the myosin had completely disintegrated. This experiment also indicates that even the skeleton substance can break into small entities. A parallel to this is found in the tropomyosin investigated by Bailey (2). Tropomyosin has almost the same amino acid composition as myosin and is considered by Bailey to be a precursor of myosin. Under certain conditions it aggregates into chains while under other conditions it breaks into small entities. Another parallel is myosin's ability to aggregate in threads.

A more systematic investigation is in progress the object of which is to determine the parts which can split from myosin and analyze them by physico-chemical methods. Guba (12) has already observed that a protin somehow involved in contraction can be split off if extracted with 0.025 M KCl. The actomyosin formed from extracted myosin and actin does not contract when ATP is added. According to Guba the protin is extremely difficult to remove when even a small quantity of actin is present.

Crystallized myosin purified with ammonium sulphate fractionation was used in the first investigation. However, this myosin gave a very poor yield of protin although the ability to contract decreases. Heart myosin proved to be better. This latter is inherently actin free after some precipitation by dilution with water. Sufficient quantities of protin can be obtained by extraction with distilled water and concentration of the water solution by ultrafiltration for purposes of physico-chemical analyses. There is a nucleoprotein in the solution which is obviously very loosely bound to myosin when actin is absent. In previous investigations a nucleoprotein was sometimes observed to be present in Edsall's solutions and even together with actin which had a sedimentation constant of about 4. This was probably the same

protin. It has actually been established that some of the substance goes into solution upon precipitation of the myosin. The fact that ammonium sulphate-precipitated myosin gives a decreased yield is due to the fact that the protin splits off during precipitation. The water-treated myosin has nearly the same precipitation curve at 0° as the first component in the myosin which precipitates at 24° (see p. 238).

ACTIN

Actin was prepared according to the method described by Straub (22). Actin obtained in this way has low viscosity and is usually designated as G-actin. In the presence of salt it readily polymerizes to F-actin with high viscosity.

In addition to the method described by Straub, F-actin can be prepared by adding ATP to Edsall's solutions and separating the F-actin by differential centrifugation. This method has not been thoroughly investigated.

G-actin cannot be studied in more detail without inactivating materials which prevent polymerization except with the electron microscope. The G-actin obtained by Straub's method is not completely pure. Many large clumps of compact actin are seen in the electron microscope. Purification of the actin by precipitation with acetone at pH 4.6 in accordance with Straub's method improves the results. G-actin almost always appears in loose aggregates (Fig. 8). The aggregation is probably significant in the process of polymerization. Straub (10) has found that actin is difficult to purify partly because it has a prosthetic group and partly because it is easily oxidized.

An attempt to determine the molecular weight by using inactivated actin did not lead to any definite result (20). The inactivating substances used were 0.5 M KI, 0.1 M KSCN, glycine, pH 9 and calgon (hexametaphosphate). In all these cases the solutions appeared to be polydisperse, often in a similar way. The sedimentation constant was about $s_0 = 3.2–3.8$; several times in the calgon solutions a sedimentation constant half this size ($s_0 = 1.6–1.7$) was observed. An attempt to determine an average molecular weight gave the value 150,000. This is about twice the value Straub found by tryptophane analysis. Since the solutions were polydisperse when a single component was expected the results indicate that the actin had either slightly polymerized or was partly damaged. Therefore the question of actin's actual molecular weight cannot yet be answered.

Fig. 8. Electron micrograph of purified G-actin.

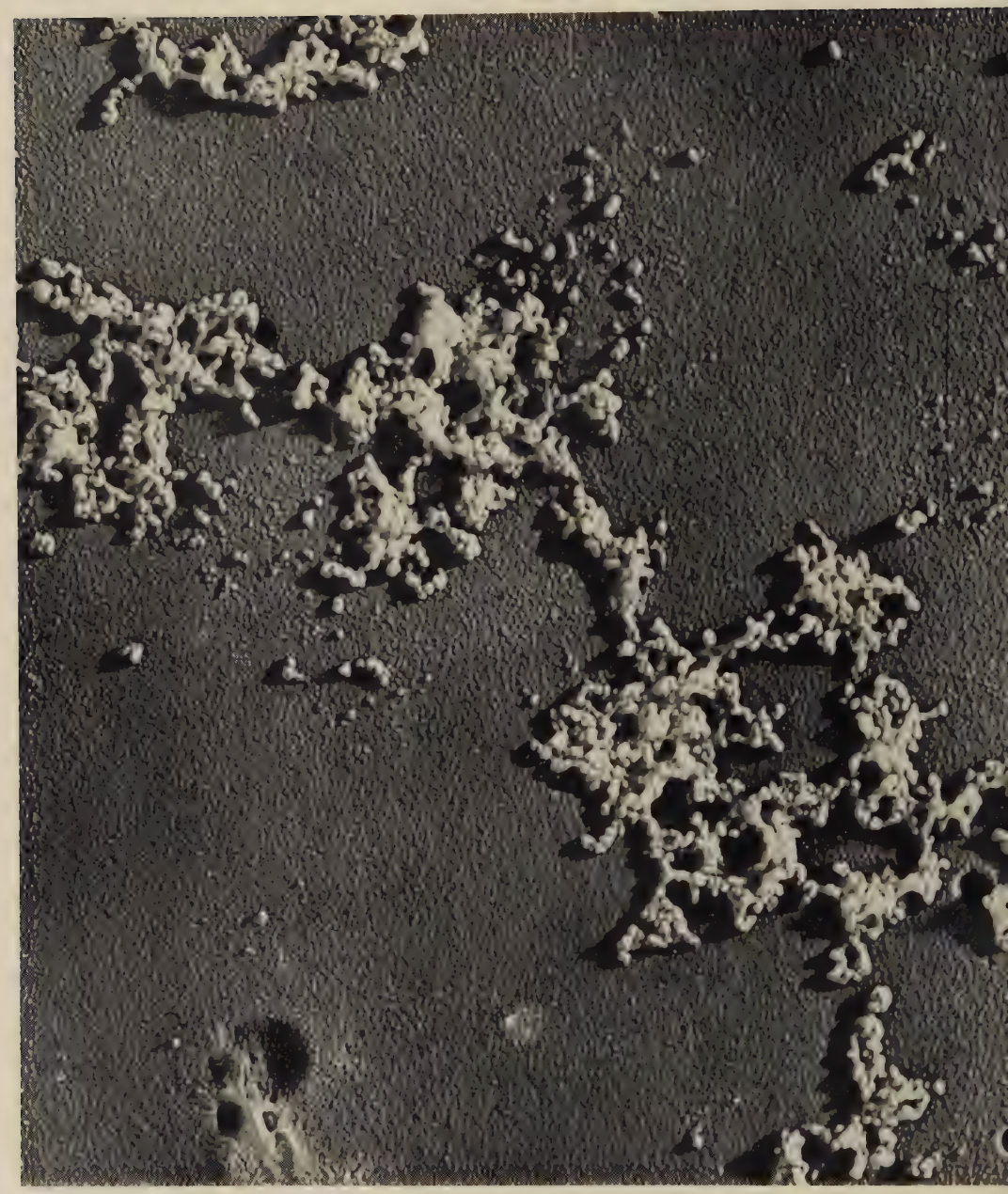


Fig. 9. Electron micrograph of heart G-actin (not purified).

Fig. 10. Electron micrograph of heart G-actin with small amount of ATP added

In connection with research on heart actin, preparations were observed in the electron microscope. It appeared that the whole amount of G-actin was aggregated into large clumps. This indicates some impurity which keeps the actin molecules together (Fig. 9). This actin polymerized badly. It was ascertained that the viscosity decreased instead of increasing when ATP was added to G-actin solutions. Thus no polymerization resulted. An investigation of G-actin with added ATP in the electron microscope showed that the actin had lost its structure and changed to an even film or protein covering the supporting membrane (Fig. 10). A complete change in the material had occurred, as was also shown by the sedimentation diagrams.

In order to purify this G-actin, precipitation was carried out at pH 4.6. Examination of the supernatant solution showed that this contains an inosin compound which passed the dialysis membrane and consequently was low molecular. Since ATP evidently catalyzed the breakdown of actin, and inosin compounds could be prepared from ATP, the dialysate from the precipitated and redissolved actin was observed. The ultraviolet absorption showed that an adenylic derivative was present. According to Banga (4) a deaminase which directly attacks ATP is found in muscle. There are definite reasons to suppose that this deaminase accompanies the actin preparations. It has been observed that an inosin compound is less formed in the usual actin preparations. Presumably, the large clumps noted in the electron microscope in that connection are actin bound together by the same type of substance as in heart actin.

Straub (10) found a prosthetic group in actin which takes part in the polymerization. This can be dialyzed, and the ability of polymerizing then disappears. Straub has not stated what this group is. It has been concluded from this investigation that it is probably the question of a low molecular weight adenylic derivative. It appears to contain ATP on the basis of physiological reactions which will be discussed later.

It should be mentioned that actomyosin prepared from Edsall's solutions of heart muscle behaved perfectly normal upon addition of ATP.

Straub (10) studied the process of polymerization by viscosity measurements. This process appears to follow the same course as for example, that of polymerization of metacrylic acid, namely first an incubation period and then a sudden polymerizing action catalyzed by Mg ions. The incubation period is a minimum in 0.1–0.15 M KCl. The relatively high molecular weight of actin indicates that it aggregates during the incubation period. The groups which Straub found to oxidize easily are involved in this part of

the process. This also explains why G-actin usually appears as aggregates when studied in an electron microscope.

F-actin has not been studied in detail. It is polydisperse and the fibers are often aggregated. The state of F-actin depends on many factors, such as pH of the solutions, the presence of salts, etc. The electrophoretic mobility of F-actin is greater than that of actomyosin and myosin.

ACTOMYOSIN

Two types of actomyosin may appear when myosin and actin are combined: G-actomyosin from myosin and G-actin, and F-actomyosin from myosin and F-actin. F-actomyosin is the only one of these which contracts. A more detailed examination of the G-actomyosin was not made. In the sedimentation diagram the presence of G-actomyosin appears as the increased polydispersity of a component which sediments as myosin.

F-actomyosin consists of long threads, often rather tightly bound together. Upon sedimentation analysis two components may be distinguished. One has gel character and sediments very rapidly; the other behaves in the same way as fiber molecule. The gel component seems to be due to an instability in the connections between myosin and actin. From the beginning Szent-Györgyi (3) has been of the opinion that the F-actomyosin in Edsall's solutions is of variable composition depending on the proportions of myosin and actin. Since this was contrary to our experience, an analysis of the composition seemed necessary. F-actin in known increasing quantities was added to a series of solutions of constant myosin content and sedimentation analyses (19) of the solutions performed. The content of myosin in the different solutions was calculated from the sedimentation diagrams. This showed that the myosin content diminished in direct proportion to the quantity of added F-actin (Fig. 11). All myosin disappeared when a definite proportion of F-actin was added, and afterwards a component which could be identified as F-actin appeared. Viscosity experiments led to a similar conclusion. These measurements are difficult to make, because of viscosity anomalies and require strictly limited experimental conditions. However, this investigation showed that there must be a definite stoichiometric relationship between the actin and the myosin in actomyosin. The determination from ultracentrifuge data gave the proportions 1 part actin and 3 parts myosin, whereas viscosity data gave 1 part actin to 2.5 parts myosin. Although it was not possible to obtain any greater precision in the determinations

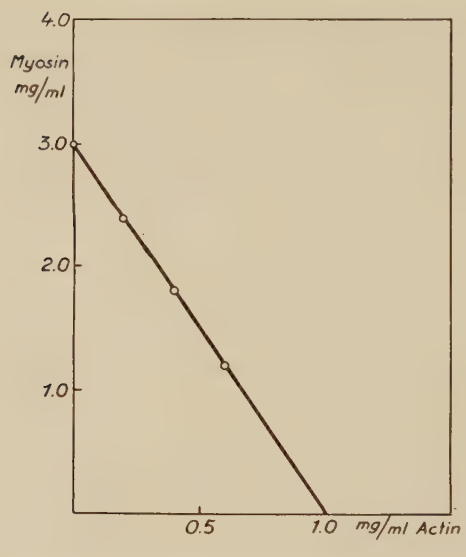


Fig. 11. Amount of myosin in solutions containing initially 3 mg/ml myosin as different amounts of F-actin are added. The amounts of myosin are calculated from the sedimentation diagrams.

because of experimental errors and difficulties in defining the substances, the investigation nevertheless shows that both actin and myosin must be definite substances. For quantitative investigations of actomyosin it is necessary that an actomyosin is chosen in which myosin and actin are mixed in such proportions that neither free myosin nor free actin is present in the solution.

SOME POINTS OF VIEW CONCERNING THE PROBLEM OF CONTRACTION

Salts play an important role in contraction. On the addition of ATP in excess to actomyosin dissolved in 0.5 M KCl, it can be observed in the sedimentation diagram that the actomyosin component disappears and that one myosin component and one F-actin component appear in its place. Actomyosin contracts when ATP is added to an actomyosin solution of low salt content, changing to a compact floccullent precipitate. Szent-Györgyi has observed that the upper limit of salt concentration where the phenomenon occurs is sharp, lying near 0.15 M KCl.

The investigations of Hermann (13) of the co-precipitation of ATP when myosin is precipitated show that ATP is not co-precipitated in pure Ca-salt solutions, and that in solutions containing $CaCl_2$ and KCl, co-precipitation of ATP increases with increasing KCl concentration and has a maximum value at a concentration somewhat above 0.1 M KCl. These determinations were made with solutions containing 5×10^{-4} M $CaCl_2$ added in increasing amounts.

Investigations of the electrophoretic mobility (9) showed that myosin in KCl solutions behaves like normal protein, with an isoelectric point near pH 5.4. In calcium and magnesium salt solutions, on the contrary, no isoelectric point was apparent. The bivalent ions are evidently very tightly bound to the myosin. In tests with solutions containing both Ca and K ions, the myosin migrated to the positive or the negative electrode, depending on the proportions of K and Ca. It was not possible to perform extensive investigations of this relationship because of the low solubility of myosin, but the combination is evidently reversible. The adsorption of ATP is determined by the relationship between the K ion concentration and the concentration of bivalent ions and probably the ATP is adsorbed to the groups which bind Ca ions. It is possible that the nucleotide part is adsorbed, since its absorption spectrum is altered when ATP is taken up by the myosin molecules.

These writers' investigations of actin are capable even at this stage of clarifying the apparent differences which have been found between the contraction of isolated actomyosin and of muscle. Actomyosin does not contract until ATP is added. According to Szent-Györgyi, this requires a definite minimum quantity, about 0.01 per cent, in order to bring about contraction in 0.15 M KCl or lower salt concentrations. Buchthal, Deutsch and Knappeis (5) found that ATP added to an isolated muscle fibril or injected intra-arterially caused an almost tetany-like contraction. They concluded that ATP is present in muscle in an inactive form which becomes activated on the addition of ATP from an external source. Rózsa (16) investigated muscle fibrils in Ringer's solution and in solutions containing 0.2–0.25 M KCl and found that the fibrils contract permanently when ATP is added. The minimum ATP required was 0.5 γ ATP per ml. If the fibril had lost its ATP through washing or rather long storage it acted like actomyosin.

The present investigations with actin indicate that ATP is partially imbedded in actin and that it can be deaminated by an enzyme which is not extracted with actomyosin in the extraction procedure. In the muscle a reac-

tion can take place between the deaminase and the muscle ATP which is affected by ATP added from outside; in this case the muscle contracts when the actin is destroyed. Actin plays a more dominant role in the contraction process than was believed before and is not merely a support substance for the myosin part. Evidently proteins which are neither actin nor myosin can play a decisive role in the course of the contraction.

It appears impossible to give a complete picture of the process of contraction with the data now available. A working hypothesis concerning the structure of actomyosin is based on the following physico-chemical data.

1) Astbury (1) has proved that the X-ray scattering of actomyosin shows considerable similarity to the scattering of keratin (at least at wide scattering angles).

2) Astbury's (15) work shows that at least slight contraction takes place in the non-crystalline part of the muscle fiber, and that the fiber axis is parallel with the length of the fiber.

3) It is possible to obtain a non-contractile F-actomyosin.

4) Certain substances usually bound to the myosin are involved in contraction.

5) F-actin is necessary for contraction and according to X-ray data there must be a change in the actin chain as well, at least when contraction is extensive.

6) Contraction is accompanied by syneresis.

The framework in myosin is probably a fiber like keratin but without S-S bonds like those which hold such fibers extended. It is possible that there are S-H bonds. If the S-S bonds in keratin are broken, these fibers can contract (1). If the S-H bonds are in some way bound in myosin and these bonds are broken by the action of ATP, the myosin fiber can contract. The contractile protins play a definite part in such a process.

The model, which we can very roughly imagine is reproduced in Fig. 12. The myosin chain is held to the actin chain; X-ray data indicate that the chains are adjacent. Where the chains are directly interconnected, there is a portion which appears in the X-ray diagram to be the crystalline part. The non-crystalline part is found where the protins join the framework substance. The initial contraction process takes place here and is not directly apparent in the X-ray diagram. This part is open and other enzymes present in the muscle can act here. It is still not known how contraction occurs, but the myosin framework probably draws together at the same time that a fundamental alteration of the structure of the actin takes place in the open part of the actin chain.

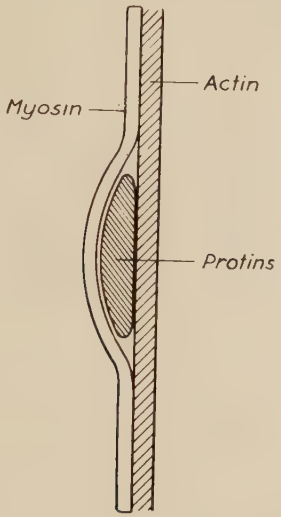


Fig. 12.

There is no point in trying to develop a more detailed hypothesis on the basis of the material available. Much work has still to be done to purify the various protins in myosin and determine their proportions and study the effect of their presence or absence before the main factors in the process of contraction will be clarified.

Muscle chemistry offers a rich field for all types of protein investigations. It was a great advance in protein chemistry when the protein molecule was defined. Within the field referred to there are many examples not only of protein molecules but also of complex combinations of protein molecules which may occur in stoichiometric proportions, and give rise to new complexes which in certain respects even have properties different from those of the protein molecules of which they are built.

These investigations were supported by a grant from the Swedish Natural Science Research Council.

REFERENCES

1. Astbury, W. T., *Proc. Roy. Soc.*, **B 134**, 363 (1947).
2. Bailey, K., *Biochem. J.*, **43**, 271 (1948).
3. Banga, I., Guba, F., and Szent-Györgyi, A., *Nature*, **159**, 194 (1947).
4. Banga, I., and Josepovits, G., *Hung. Acta Physiol.*, **I**, 90 (1947).
5. Buchthal, F., Deutsch, A., and Knappeis, G. G., *Acta Physiol. Scand.*, **11**, 524 (1946).
6. Csápo, A., Erdös, T., Naeslund, J., and Snellman, O., *Biochim. et Biophys. Acta* (in press).
7. Derrien, Y., *Trav. Soc. Chim. Biol.*, **XXVI**, 1091 (1944).
8. Engelhardt, W. A., and Ljubimova, M. N., *Nature*, **144**, 669 (1939).
9. Erdös, T., and Snellman, O., *Biochim. et Biophys. Acta*, **2**, 642 (1948).
10. Feuer, G., Molnár, F., Pettko, E., and Straub, F. B., *Hung. Acta Physiol.*, **I**, 150 (1948).
11. Gelotte, B., (unpublished).
12. Guba, F., (quoted in (24)).
13. Hermann, V. Sz., *Hung. Acta Physiol.*, **I**, 21 (1946).
14. Needham, J., Kleinzeller, A., Miall, M., Dainty, M., Needham, D. M., and Lawrence, A. C. S., *Nature*, **147**, 766 (1940).
15. Perry, S. V., Reed, R., Astbury, W. T., and Spark, L. C., *Biochim. et Biophys. Acta*, **2**, 674 (1948).
16. Rózsa, G., *Hung. Acta Physiol.*, **I**, 16 (1946).
17. Snellman, O., and Erdös, T., *Biochim. et Biophys. Acta*, **2**, 650 (1948).
18. —— *Biochim. et Biophys. Acta*, **2**, 660 (1948).
19. —— *Biochim. et Biophys. Acta*, **3**, 523 (1949).
20. Snellman, O., Erdös, T., and Tenow, M., *Proc. 6th Int. Congr. Exp. Cytology*, 247 (1949).
21. Snellman, O., and Tenow, M., *Biochim. et Biophys. Acta*, **2**, 384 (1948).
22. Straub, F. B., *Studies Inst. Med. Chem. Univ. Szeged*, **II**, **3** (1942), **III**, **23** (1943).
23. Szent-Györgyi, A., *Acta Physiol. Scand. IX*, Suppl. XXV (1947).
24. —— *Muscular Contraction*, New York, 1947; *Nature of Life*, New York, 1948.
25. Tenow, M., To be published.

DOUBLE ORIGINE DES RIBONUCLEOPROTEINES CYTOPLASMIQUES DANS L'OOCYTE DE GLOMERIS MARGINATA

E. FAURÉ-FREMIET, H. COURTINES et H. MUGARD

Collège de France, Paris

Reçu le 8 décembre 1949

I. HISTORIQUE

J. Lubbock (8), E. G. Balbiani (1), C. Bisogni (2), B. Nemec (9) décrivent dans les jeunes oocytes de différents Myriapodes, et plus spécialement de Diplopodes, la présence dans le cytoplasma, au contact de la vésicule germinative, d'une sorte de coiffe, ou d'épaississement localisé, qui s'accroît ultérieurement en forme de boudin, puis se fragmente et se désagrège au début de la vitellogenèse.

A. Lecaillon (7) écrit, à propos de l'œuf du Chilognate *Polyxenus lagurus* « dans le cytoplasma ovulaire apparaît de très bonne heure une matière différenciée spéciale que je désignerai simplement sous le nom de substance cytochromatique ». Lecaillon, constatant l'apparition et le développement de cette substance autour de la vésicule germinative, compare cette structure à celles observées par B. Nemec (9) chez *Polyzonium*, *Blaniulus*, *Polydesmus* etc., et aux corps vitellins décrits chez l'Arachnide *Pholcus* par Jatta (1882) et par van Bambeke (1898). Il constate encore que sa substance cytochromatique présente les mêmes affinités tinctoriales que les nucléoles.

E. Fauré-Fremiet (4) interprète faussement la masse vitellogène périnucléaire, dans l'oocyte de *Julus terrestris*, comme une masse mitochondriale.

A. Koch (6), étudiant la croissance de l'œuf des Chilopodes, apporte une description détaillée des coiffes et des capes safranophiles formées autour de la vésicule germinative, chez *Geophilus* en particulier, et discute longuement sur le rôle du noyau dans ce processus.

Enfin, C. Jacquiert (5), dans une belle étude sur l'oogenèse chez les Araignées, montre que le « corps vitellin » d'*Araneus* est constitué de vacuoles et de chondriosomes, mais que sa masse acidophile reste très nettement délimitée du cytoplasma basophile qui l'entoure, et se disloque au début de

la vitellogenèse; « le corps vitellin de *Julus* décrit par E. Fauré-Fremiet semble identique, écrit-il, à celui d'*Araneus* ».

Chez *Pholcus*, le « corps vitellin » apparaît comme une masse en croissant, plus réfringente que le cytoplasma, entourée de mitochondries et de vacuoles; mais, ajoute Jacquiert, « les constituants cytoplasmiques ne prennent aucune part à sa formation ».

Il reste bien difficile, à partir de tous ces faits intéressants, de préciser la signification cytologique des corps périnucléaires décrits par les auteurs chez les Chilopodes.

II. ASPECTS MORPHOLOGIQUES DE LA VITELLOGENESE CHEZ L'OOCYTE DE GLOMERIS MARGINATA

Les femelles de *Glomeris marginata* recueillies au mois d'avril dans les environs de Paris montrent tous les stades de la vitellogenèse. Les oocytes ont été étudiés à l'état frais, par dissection et dissociation des ovaires dans une solution physiologique; après fixation et traitement approprié ils ont été examinés soit *in toto*, soit sur coupes.

Les oocytes dont le diamètre moyen est de 30 à 40 μ montrent un cytoplasma très fortement basophile, fixant énergiquement le bleu de toluidine, d'apparence granuleuse homogène, dans lequel les mitochondries sont plus ou moins également dispersées.

Chez les oocytes dont le diamètre moyen dépasse 40 μ, on constate l'apparition de calottes périnucléaires acidophiles. Plus ou moins étendues, épaisses de quelques μ, elles coiffent la vésicule germinative et paraissent être au contact immédiat de sa paroi, toujours visible comme une fine membrane. Aucune relation ne paraît exister entre ces masses intracytoplasmiques et le nucléole.

Aux stades ultérieurs (Fig. 1), les masses acidophiles s'épaississent et s'accroissent irrégulièrement en repoussant le cytoplasma basophile, dont elles peuvent aussi inclure des parties, et dans lequel elles se fragmentent en masses ovoïdes de volume variable; aucun mélange ne s'effectue entre ces deux constituants cellulaires, dont l'interface est toujours nettement marquée. La répartition des mitochondries n'est plus quelconque; les techniques adéquates (Altmann p. ex.) montrent que tous les chondriosomes se trouvent exclusivement localisés à l'interface du cytoplasma et des masses acidophiles, faisant à ces dernières un revêtement granuleux plus ou moins régulier et semé de petits amas (Fig. 2).

Sur les coupes colorées au bleu de toluidine-éosine, l'aspect des oocytes est curieusement caractérisé par les nappes rose pur, aux contours nets, entourées par le cytoplasma bleu intense (Fig. 1).

Lorsque le diamètre des oocytes atteint et dépasse une centaine de microns, l'aspect change; les masses acidophiles se fragmentent en petites gouttelettes dont les contours s'estompent bientôt, comme si leur substance commançait à se mélanger avec le cytoplasma environnant dont la basophilie s'atténue (Fig. 3). En même temps, les mitochondries semblent se multiplier et se dispersent, de plus en plus nombreuses, dans toute la masse cytoplasmique.

Ce stade précède immédiatement la vitellogenèse proprement dite; celle-ci se traduit, lorsque les masses acidophiles ont presque complètement disparu, par l'apparition de globules réfringents, constitués par des substances protéiques aux réactions tinctoriales complexes, et de petits globules graisseux. Les globules vitellins protéiques augmentent ensuite en nombre et en dimensions jusqu'à la fin de la croissance de l'oocyte.

Ces faits montrent que l'on peut distinguer, chez l'oocyte de *Glomeris*, une période de prévitellogenèse, caractérisée par le développement des corps acidophiles jusqu'à leur dislocation finale; puis une période de vitellogenèse proprement dite, caractérisée par la formation des globules protéiques. Laissant de côté la question de savoir si la notion de « corps vitellin de Balbiani » peut admettre une définition générale, le problème de la composition et de la signification biochimique des masses acidophiles reste posé.

III. PROPRIETES PHYSIQUES DES CORPS ACIDOPHILES

La substance constituant les corps acidophiles, considérée indépendemment des inclusions cytoplasmiques que ceux-ci peuvent enrober, apparaît très homogène.

A l'éclairage sur fond noir, avec un condensateur cardioïde, cette substance paraît obscure, tandis que son interface cytoplasmique apparaît granuleuse et brillante par le fait de l'accumulation des mitochondries et, probablement, de granules réfringents de petite taille (Fig. 4).

En lumière polarisée, les corps acidophiles restent parfaitement isotropes.

Ces corps peuvent être séparés du cytoplasma de l'œuf par microdissection à l'état frais; on constate qu'ils présentent la consistance d'une gelée rigide; piqués avec une aiguille, ils restent sans déformation empalés sur celle-ci, tandis qu'une faible pression les écrase.

Une élévation de température suffisante provoque une sorte de coagulation, accompagnée d'une légère rétraction volumétrique.

Les solutions M/10 de LiCl, KCl, $CaCl_2$ et de Sulfocyanure de K, ne provoquent aucune modification apparente; les solutions concentrées de $CaCl_2$ provoquent une légère rétraction, de nature probablement osmotique. LiCl à 10 % donne un léger gonflement. L'aspect négatif de ces différents essais n'apporte aucune indication caractéristique.

Les colorants vitaux utilisés en solution physiologique, n'apportent pas plus d'information; le rouge neutre colore légèrement et lentement en rose les corps acidophiles; le vert janus les teinte légèrement; le violet dahlia les colore plus nettement en violet rougeâtre; le bleu de crésyl brillant est sans effet.

IV. PROPRIETES CHIMIQUES

L'effet négatif des solvants et des colorants des graisses (rouge Sudan, bleu BZL) élimine aussitôt l'idée d'un constituant lipidique des corps acidophiles.

L'absence de coloration métachromatique, la non fixation du mucicarmin, la teinte jaune communiquée par l'iode, le résultat négatif de la technique de Bauer, permettent d'exclure l'idée d'un constituant glycoprotéique ou polysaccharidique.

Par contre la coloration brun rouge très nettement communiquée à ces corps acidophiles par le réactif de Millon-Bensley, indique la présence de protéines, à une concentration nettement plus forte que dans le cytoplasme environnant (Fig. 5).

La réaction de Feulgen est négative.

La coloration au vert de méthyle-pyronine de Unna et J. Brachet colore les corps acidophiles en un rouge notablement moins intense que pour le cytoplasma basophile. Après action de la ribonucléase la colorabilité par la pyronine disparaît à la fois pour le cytoplasma basophile et pour les corps acidophiles. De ce fait la présence de ribonucléoprotéines paraît hautement probable dans le cytoplasma basophile, et possible dans les inclusions d'origine périnucléaire, malgré leur acidophilie et leur affinité pour l'éosine.

La nature protéique des corps acidophiles est encore démontrée par leur disparition complète et rapide au cours de la digestion trypsique des oocytes, effectuée *in vitro*, sous le microscope, à la température de 37° C.

Par contre, l'action de la pepsine chlorhydrique à 37° provoque la séparation progressive, dans les masses acidophiles, d'un résidu non digéré;

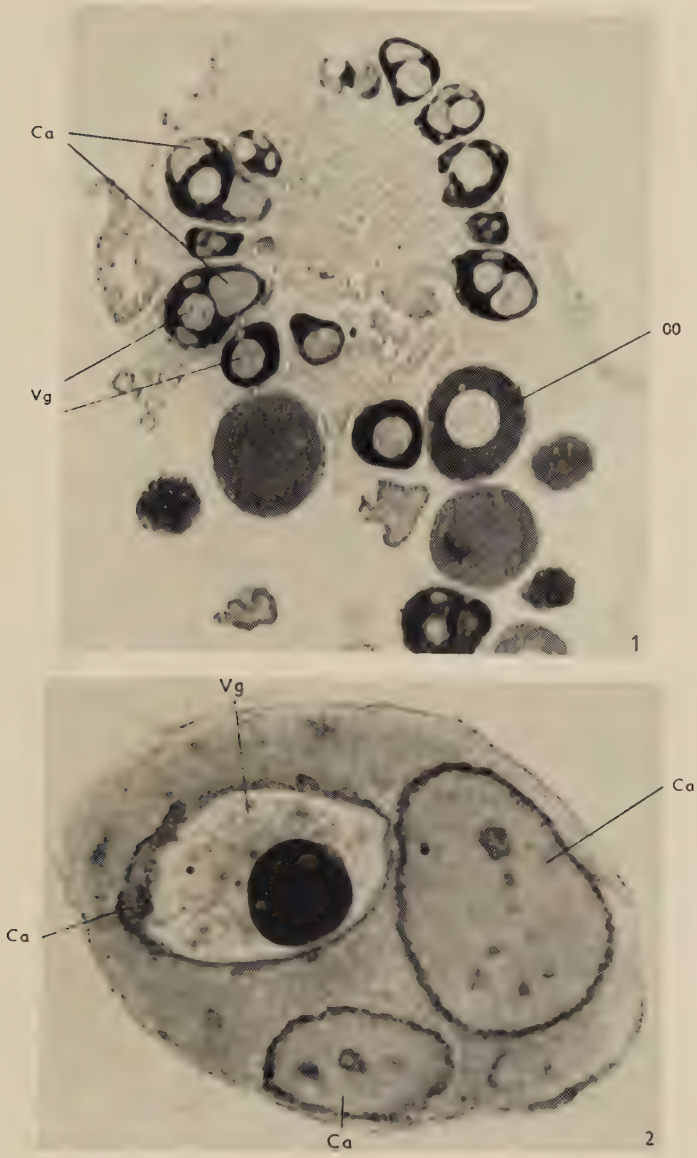
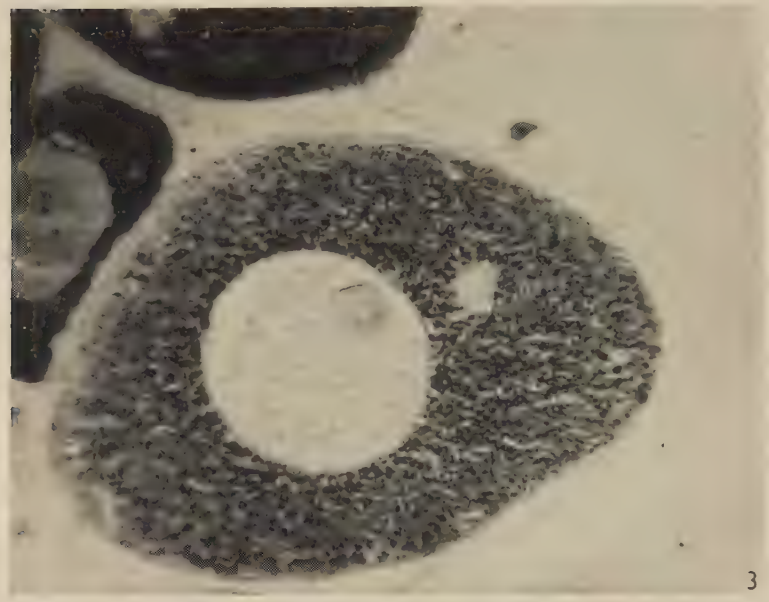


Fig. 1. Portion d'un ovaire de *Glomeris* coloré au bleu de toluidine-éosine; les jeunes oocytes montrent une coloration intense du cytoplasma; vésicules germinatives (Vg) et corps acidophiles (Ca) clairs. Les oocytes au début de la vitellogenèse (00) sont caractérisés par leur cytoplasma uniformément granuleux.

Fig. 2. Oocyte coloré par la technique de Altmann; les mitochondries sont rassemblées en couche dense à la surface des corps acidophile (Ca) et de leurs inclusions; quelques uns de ceux-ci commencent à se former au contact de la vésicule germinative (Vg).



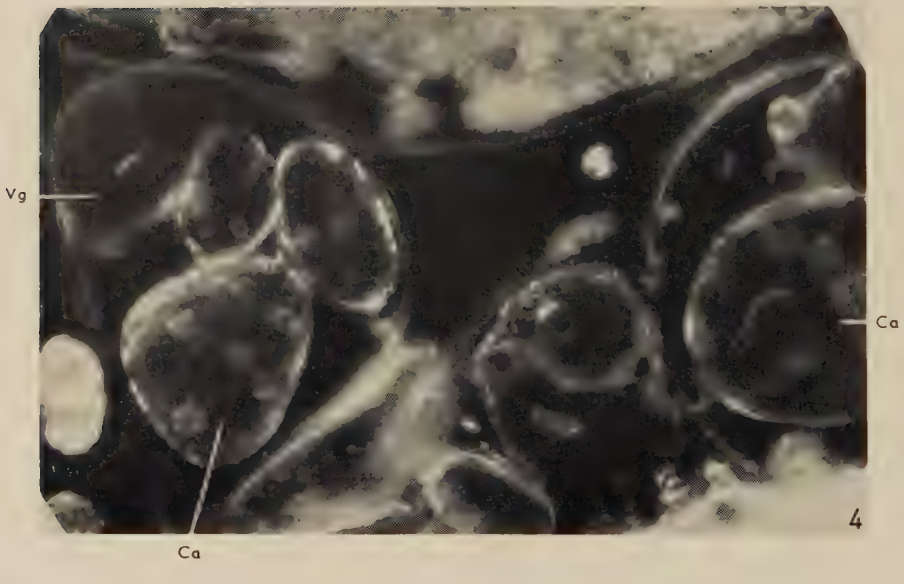


Fig. 3. Oocyte au premier stade de la vitellogenèse montrant la dispersion du matériel acidophile et l'aspect granuleux du matériel basophile; coloration bleu de toluidine-éosine.

Fig. 4. Oocytes examinés sur fond noir (condensateur cardioide); la surface des corps acidophiles (Ca) et de leurs inclusions cytoplasmiques apparaît brillante et granuleuse; Vg = vésicule germinative avec nucléole.

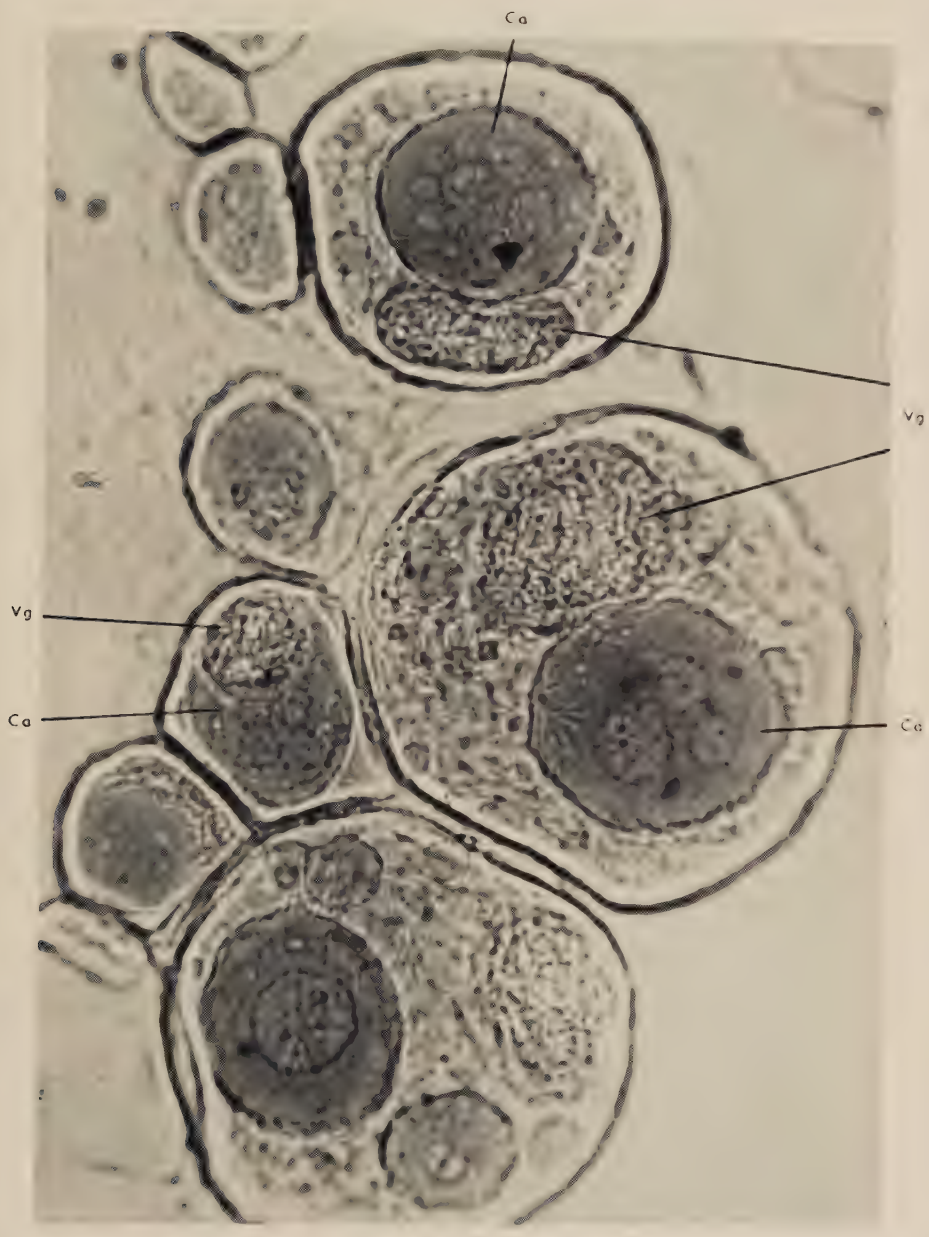


Fig. 5. Oocytes après action du réactif de Millon-Bensley; les corps acidophiles (Ca) sont beaucoup plus colorés que le reste du cytoplasma. Vg = vésicule germinative.

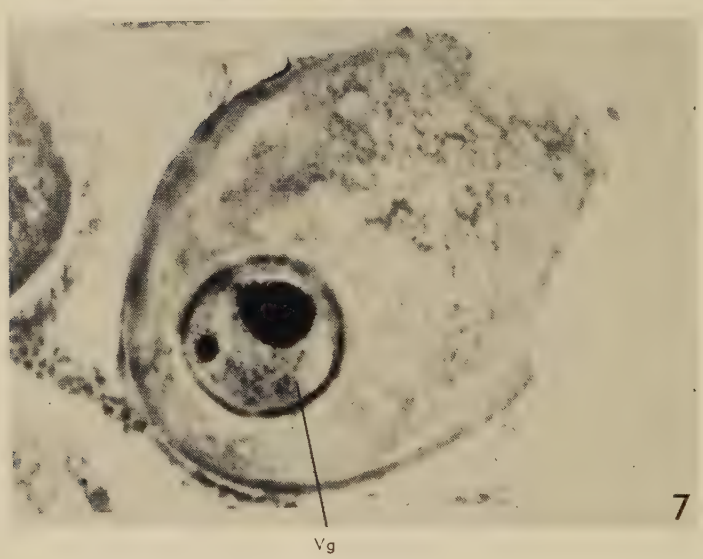


Fig. 6. Effet de la digestion pepsique; les corps acidophiles sont profondément altérés et des gouttelettes réfringentes (g), apparemment visqueuses, se séparent de la masse initiale et se fusionnent en globules vacuolisés.

Fig. 7. Détection de la phosphatase alcaline par la méthode de Gomori; dans la vésicule germinative (Vg) le nucleole montre une réaction fortement positive.

celui-ci se condense sous forme de masses irrégulières, vacuolisées, très réfringentes, rappelant l'aspect d'un nucléole (Fig. 6); ces masses se colorent fortement par la pyronine et disparaissent sous l'effet de la ribonucléase.

Ce fait significatif indique la présence de ribonucléoprotéines qui se séparent progressivement des protéines digérées par la pepsine. Ce sont donc ces protéines qui masquent les affinités des composés ribonucléiques en leur superposant une forte acidophilie, indice de leur caractère basique.

Il convenait de rechercher quelques caractères des histones. Utilisant les résultats obtenus par A. E. Mirsky et A. W. Pollister, voir (10), nous avons essayé, après fixation au liquide de Carnoy, une coloration au bleu d'aniline légèrement alcalinisé, qui a coloré d'une manière assez élective les corps acidophiles; et l'on a constaté d'autre part, que l'action prolongée du réactif de Millon provoque une altération marginale de ces corps et leur confère un aspect réticulé. Ces indications, dont il ne faut pas exagérer la signification, seraient d'accord avec la présence de protéines basiques du type des histones.

V. LA REPARTITION DES RIBONUCLEOPROTEINES DANS L'OEUF DE GLOMERIS

Les faits qui viennent d'être exposés montrent que le cytoplasma de l'oocyte de *Glomeris* est très fortement basophile dès les premiers stades de son accroissement; comme sa colorabilité par le bleu de toluidine et la pyronine disparaît entièrement après action de la ribonucléase, on est en droit de conclure à l'existence, chez cet œuf comme chez beaucoup d'autres, de ribonucléoprotéines proprement cytoplasmiques.

Quelques essais ont montré que le constituant basophile peut être rassemblé par une forte centrifugation, ce qui permettrait de supposer sa liaison avec des éléments particulaires de petite taille, tels que les microsomes de Claude et de J. Brachet.

D'autre part, les corps acidophiles qui apparaissent au cours de la période de prévitellogenèse, contiennent, eux aussi, des ribonucléoprotéines, mais les protéines basiques qui leur sont associées masquent leurs propriétés tinctoriales.

L'apparition des corps acidophiles à la surface de la vésicule germinative, en contact étroit avec sa membrane, laisse supposer leur origine nucléaire, conformément au schéma proposé par Caspersson. Il ne semble pas que le nucléole participe directement à leur formation, malgré quelques analo-

gies de colorabilité. Le nucléole donne par ailleurs une forte réaction de la phosphatase alcaline (Fig. 7), comme celui de l'œuf de Tégénaire étudié par J. R. G. Bradfield (3), et cette réaction est négative au niveau des corps acidophiles.

Il reste à supposer que les ribonucléines et les protéines basiques diffusent à travers la membrane nucléaire et forment au contact du cytoplasma un système coacervé non miscible, dont l'interface fixe temporairement les mitochondries et d'autres granulations.

En d'autres termes, les ribonucléines présentes dans le cytoplasma de l'oocyte en prévitellogenèse, auraient une double origine, l'une proprement cytoplasmique et l'autre nucléaire.

Comme dans tous les autres cas étudiés par J. Brachet, la richesse du cytoplasma en composés ribonucléiques apparaît liée à la synthèse massive de protéines (du type vitellines) dont l'oocyte est le siège au cours de la période de vitellogenèse.

Comparant les structures observées chez l'œuf de *Glomeris* avec les nombreuses observations des auteurs cités au début de ce travail, il semble probable que la double origine des composés ribonucléiques soit assez générale chez les Myriapodes et se retrouve chez une Arachnide dans le cas de *Pholcus*. Cela ne signifie aucunement que les « corps vitellins » d'origine nucléaire présents dans les oocytes de ces diverses formes soient exactement comparables à d'autres corps vitellins, contenant aussi, comme ceux des œufs de Tégénaire d'après J. R. G. Bradfield, des ribonucléoprotéines, l'origine cytoplasmique de nombre d'entre eux paraissant évidente d'après les recherches de C. Jacquiert.

RESUMÉ

Le cytoplasma du jeune oocyte de *Glomeris marginata*, intensément basophile, est riche en ribonucléoprotéines, liées probablement à des microsomes.

Au cours de la prévitellogenèse, des corps acidophiles apparaissent dans le cytoplasma, au contact de la vésicule germinative; diverses réactions microchimiques montrent qu'ils contiennent des ribonucléoprotéines, dont les affinités tinctoriales sont masquées par la présence de protéines basiques.

Au cours de la vitellogenèse proprement dite, les deux sortes de constituants ribonucléoprotéiques disparaissent tandis que les globules vitellins protéiques commencent à se constituer.

Les corps acidophiles correspondent à ce que l'on a décrit chez d'autres

Myriapodes et chez quelques Arachnides sous le nom de « corps vitellins »; l'hypothèse de leur origine nucléaire (par diffusion à travers la couche limitante de la vésicule germinative) est vraisemblable; leurs affinités tinctoriales les rapprochent des nucléoles, mais, contrairement à ces derniers, les corps acidophiles ne contiennent pas de phosphatase alcaline.

Je ne pense trahir ni la mémoire de Maurice Parat, ni l'amitié de Hope Hibbard et de Georges Nichita, en reprenant aujourd'hui avec H. Courtines et H. Mugard l'étude de l'oocyte de *Glomeris*, entreprise autrefois avec eux; les circonstances n'ont pas permis l'achèvement de ce travail collectif auquel Jacquiert (5, p. 97 et 98) a fait allusion en 1936 et, depuis lors, les techniques se sont renouvelées et les problèmes cytologiques ont changé d'aspect.

INDEX BIBLIOGRAPHIQUE

1. Balbiani, E. G., *Zool. Anz.*, 156 (1883).
2. Bisogni, C., *Intern. Monatschr. f. Anat. u. Physiol.*, **12** (1895).
3. Bradfield, J. R. G., *Exper. Cell Research. Sup.* **1**, 338 (1949).
4. Fauré-Fremiet, E., *Arch. Anat. Microsc.*, **11**, 457 (1910).
5. Jacquiert, C., *Recherches sur l'oogenèse des Araignées et les corps vitellins de Balbiani*, 154, Paris (1936).
6. Koch, A., *Zeitschr. f. Zellforsch. u. mikr. Anat.*, **2**, 293 (1925).
7. Lecaillon, *Bull. Soc. Philomath.*, 9ème S. **5**, 70 (1902).
8. Lubbock, J., *Phil. Transact.* (1861).
9. Nemec, B., *Anat. Anz.*, **13**, 309 (1897).
10. Pollister, A., and Ris, H., *Cold Spring Harbor Symp. on Quant. Biol.*, **XII**, 147 (1946).

PROTEOLYTIC ACTIVITY IN SEA URCHIN GAMETES

I. ACTIVITY IN UNTREATED EXTRACTS

GUNNAR LUNDBLAD

The Wenner-Gren Institute for Experimental Biology, Stockholm

Received December 20, 1949

The basis for our knowledge concerning the mechanism of fertilization and artificial activation of the egg has been laid by Loeb (5) and Lillie (4). According to Runnström (10, 11, 12) the incitement of development of the fertilized egg is due to an activation of the enzymes in the egg. The present writer (6) has demonstrated that this activation occurs *in vivo* as far as proteolytic enzyme is concerned. A full report of these investigations all made on sea urchin material, including studies of a protease found in extracts from spermatozoa and proteolytic activity in jelly coat solutions, is given in this paper.

Recently Woodward (16) has shown proteolytic activity in cytoplasmic granules of unfertilized *Arbacia* eggs, using Anson's method (1) for the assay.

Experiments on activation *in vitro* of the egg enzyme will be given in a later report.

MATERIAL AND EXPERIMENTAL METHODS

The biological material, eggs and sperms from sea urchins, for the main part from the species *Paracentrotus lividus* and *Arbacia lixula*, was collected in the Gulf of Naples in the winter and spring 1948 and 1949.

The material was lyophilized, i.e. shellfrozen and vacuumdried without melting, and kept in a dry state in the cold. Extractions were made in the cold (+3° C) for about three hours and the extracts centrifuged, dialysed for 24 hours and centrifuged again. The centrifuged extracts were kept in a frozen state at —20° C and thawed immediately before use. The total nitrogen was determined according to micro Kjeldahl. All determinations of pH were made by the glass electrode.

Because of the weak enzymic activity of the extracts, their liquefying effect upon gelatin was measured by a viscosimetric method. Viscosimetric determinations of enzymic activity on gelatin had already been used by Spriggs (14). Later Northrop

(8, 9) introduced the method again. The gelatin (U. S. P. granular from Fischer Scientific Co., Pittsburg, Penn., and Eimer & Amend, New York, N. Y.) was used in 4.0 per cent solutions with 0.005 per cent merthiolate (sodium ethyl mercuri thiosalisylate, Lilly) as sterilizing agent. The merthiolate does not seem to interfere with the enzymic activities. Each sample consisted of 1.0 ml extract and 3.0 ml gelatin solution and in every experiment a control with boiled extract was run. The measurements were carried out in Ostwald viscosimeters at 35.5° C (cf. Northrop (9)) and the activities were calculated according to a formula by Hultin (2), also used by the present writer (7).

Hultin's formula: $$A^{t^\circ} = J \cdot c^2{}_s \cdot \frac{d\frac{1}{\eta_{sp}}}{dt}$$

where A = the enzymic activity at t° C (units per gram) in the reaction mixture,
J = a factor for the influence of pH,
c = the concentration of the substrate in the reaction mixture (gram substrate per gram solution),
η_{sp} = specific viscosity and
t = time in seconds

The inverse values of specific viscosity are plotted against time and the slope of the line $d\ 1\ (\eta_{sp})/dt$ is estimated. The viscosimetric method has advantages as well as limitations. The principal advantages are the sensitiveness which surpasses all other methods and the possibility of following the course of the reaction the whole time. The main disadvantage is the variation of the original specific viscosity at different pH of the gelatin. Therefore considerable corrections must be done in these cases.

PROTEOLYTIC ACTIVITY IN SPERM EXTRACTS

The dry material was extracted with distilled water or acetic acid. Table I shows the activities of some different extracts. *Arbacia* is more active than *Paracentrotus*. The total nitrogen content is less in acetic acid extracts than in water extracts but the activity calculated on gram nitrogen is greater in acetic acid extracts, which shows that the higher nitrogen content in water extracts derives from non-enzymic proteins.

The whole enzymic activity in *Paracentrotus* is found in the fraction below 50 per cent ammonium sulfate saturation.

In order to give an idea of the activity of this enzyme a comparison is made with that of crystalline trypsin measured in the same way. Trypsin is more than one million times stronger, calculated on initial dry weight, and about a hundred thousand times stronger, calculated on the nitrogen content in the enzyme solutions.

TABLE I

Proteolytic activity in sperm extracts.
Temp. 35.5° C. S–w: sea water.

Species	Exp. No	Batch	% Initial dry weight	Extract medium	Dial medium	% N in extract	Age of extr. at measurement	ph	Activity of extract	
									A/ml	A/g N
Psammechinus..	1	—	10.0	H_2O	—	—	Freshly prepared	6.25	33.40×10^{-9}	—
Paracentrotus ..	2	Pa 0	»	»	—	—	» »	»	12.70 »	—
Arbacia.....	3	A 1	5.0	»	S–w	0.037	4 days	6.50	13.20 »	35.60 ×
»	4	A 2	»	HAc M/10	»	0.0097	7 »	»	6.40 »	65.60
Paracentrotus ..	5	Pa 1	»	H_2O	»	0.058	4 »	»	1.20 »	2.10
»	6	Pa 2	»	HAc M/10	»	0.0095	7 »	»	1.24 »	13.10
Arbacia.....	7	A 1	»	H_2O	»	0.037	4 months	»	11.00 »	29.70
»	8	A 2	»	HAc M/10	»	0.0097	4 »	»	5.20 »	53.60
Paracentrotus ..	9	Pa 1	»	H_2O	»	0.058	4 »	»	5.40 »	9.30
»	10	Pa 2	»	HAc M/10	»	0.095	4 »	»	0.80 »	8.40
Arbacia.....	11	A 3	»	H_2O	»	0.031	5 days	»	1.35 »	4.35
»	12	A 4	»	»	»	0.057	1 month	6.93	4.27 »	7.48
»	13	A 5	2.5	»	»	0.012	2 days	6.50	10.80 »	90.00
»	14	A 6	»	HAc M/10	»	0.010	2 »	»	2.04 »	20.40
Paracentrotus ..	15	Pa 3	»	H_2O	»	0.015	2 »	»	1.68 »	11.20
Arbacia.....	16	A 5	»	»	»	0.012	19 »	7.50	18.10 »	151.00
Trypsin, cryst. .			0.01	»	»	0.003	Freshly prepared	6.40	47.80 10^{-6}	15.90

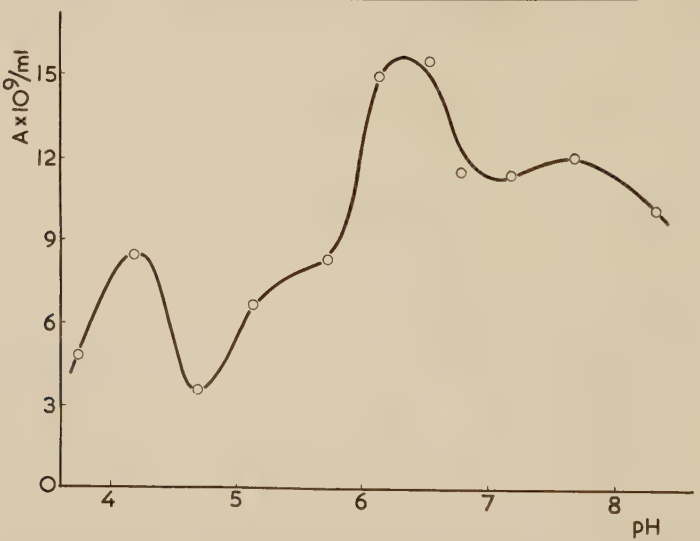


Fig. 1. Proteolytic activity of Arbacia sperm extracts. Corrections made for the pH dependence of the specific viscosity of the gelatin.

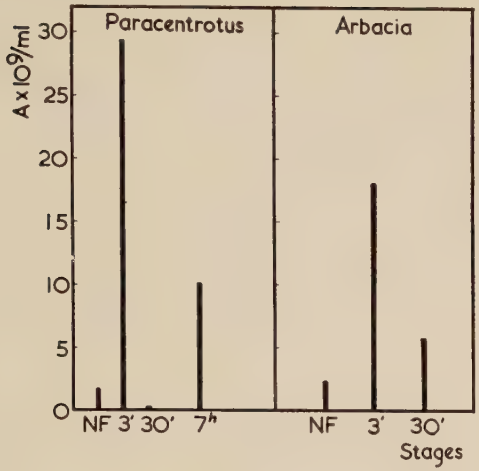


Fig. 2. Proteolytic activity of eggs at different stages. NF: unfertilized eggs.

Fig. 1 shows the pH dependence of the proteolytic activity in extracts from *Arbacia* sperm in the pH interval 3.75–8.45. The material had been stored in the cold for ten months and it was extracted with distilled water and dialysed against sea water (38 pro mille). The initial dry weight of sperm material was 5.0 per cent and the nitrogen content in the extract was 0.031 per cent. Two different series were run and the average values taken. Corrections for the activity of the enzyme at the different pH's tested were made so that the inverse value of the initial specific viscosity is put as the factor J in the formula. There is a minimum at pH 4.8 (the I. E. P. for gelatin is 4.86) and two maxima, one at pH 4.20 and another at pH 6.40 (ca). On the acid side the decrease in activity is steeper than on the alkaline side. The activity at pH 6.55 is $A^{35.5°} = 15.3 \times 10^{-9}$ per ml extract and 49.4×10^{-6} per gram nitrogen in the extract.

ACTIVITY OF EGG EXTRACTS

All egg material was extracted with distilled water. Fig. 2 shows the activities of some fresh preparations. The egg material was shellfrozen at different times after fertilization (full membrane elevation). To the unfertilized eggs the same amount of sperm was added after freezing. The pH was 6.25 and the initial dry weight 1.8 per cent. We see a strong increase in enzymic activity 3 minutes after fertilization compared with the activity in extracts

Table II

Proteolytic activity in extracts from abnormal eggs.
Arbacia eggs from two different batches I and II, all extracts dialysed against sea water. Temp. 35.5° C. NF: unfertilized eggs.

Batch	Stage	Extract medium	% N in extract	pH	Activity of extract	
					A/ml	A/g N
I	NF	M KCl	0.064	6.60	14.30×10^{-9}	22.40×10^{-6}
I	F 3′	»	0.092	»	1.70 »	1.85 »
II	NF	H_2O	0.037	7.05	1.74 »	4.70 »
II	F 5′	»	0.028	»	1.24 »	4.43 »
II	F 10′	»	—	»	0	0
II	NF	M KCl	0.052	»	2.18 »	4.19 »
II	F 5′	»	0.034	»	1.24 »	3.65 »
II	F 10′	»	—	»	0	0

from unfertilized eggs. This high activity is strongly reduced in *Arbacia* 30 minutes after fertilization and in *Paracentrotus* the activity is almost abolished so that the value is less than in extracts from unfertilized eggs. The activity which appears in unfertilized eggs may be due to an activation by freezing (cf. Kiermeier (3)) or by the influence of ions during extraction. Controls show that the quantity of sperms injected for fertilization does not contribute to any activity.

Whether the proteolytic activity which appears in the 7 hours stage derives from the same zymogen which gives the activity 3 minutes after fertilization is not yet established.

These spontaneous activations *in vivo* occuring after fertilization are found only in easily fertilized material. When there were even small difficulties in fertilization or if the embryonal development was abnormal these activity relations were not valid. Table II gives the values of some experiments with abnormal material.

The pH dependence of the proteolytic activity of fertilized *Arbacia* eggs 4.5 minutes after fertilization is shown in Fig. 3. The pH range is between 4.3 and 8.6. The material was stored 4 months in the cold, before it was extracted with distilled water and dialysed against sea water. The initial dry weight extracted was 2.5 per cent and the total nitrogen content in the extract was 0.065 per cent. The ordinate gives the activity per ml extract at 35.5° C. At pH 6.45, for example, the activity $A = 1.98 \times 10^{-9}$, calculated per gram nitrogen in the extract $A = 3.05 \times 10^{-6}$. The curve shows a minimum

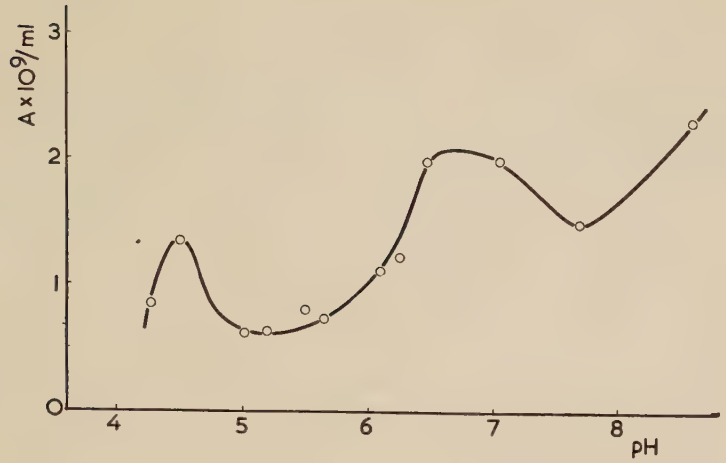


Fig. 3. Proteolytic activity of Arbacia eggs 4 $^1/_2$ minutes after fertilization. Corrections made for the pH dependence of the specific viscosity of the gelatin.

at pH 5.2 and a maximum at pH 6.5. On the acid side there is a steep decrease but on the alkaline side we find a rather steep increase in proteolytic activity.

PROTEOLYTIC ACTIVITY OF JELLY COAT SUBSTANCES

During studies of the jelly coat as a substrate for a supposed lytic factor in sperm extracts, the rather astonishing discovery was made that jelly coat solutions possessed liquefying activity on gelatin. According to Vasseur (15) the jelly coat is a mucopolysaccharide with a protein content of 20–25 per cent.

The jelly coats from unfertilized eggs were dissolved in slightly acidified sea water (pH 5.5), the solution was centrifuged to get rid of suspended eggs and then precipitated with ethanol. The precipitate was dissolved in a small volume of distilled water, dialysed against running tap water and lyophilized. From this dried material, which was stored at + 2° C, extracts were made and activity measured viscosimetrically in the usual way. In order to confirm the gelatin liquefying activity some experiments were made with haemoglobin as substrate according to Anson's method (1). The results of these measurements which also showed proteolytic activity and of the viscosimetric determinations can be seen in Table III.

Now it would perhaps seem appropriate to ascribe this proteolytic activity

TABLE III

Proteolytic activity of jelly coat solutions.

H. U.: activity measured upon gelatin and calculated according to Hultin's formula. A. U.: activity measured upon haemoglobin by Anson's method and calculated for 1 hour's digestion at 35.5° C. Batches III, V and VI are dried material stored 1 1/2 years at +2° C. In batch IV the jelly coat was never precipitated, the solution was stored at +2° C. In experiment 7 the extract was dialysed against distilled water. Extracts were always stored at −20° C.

Species	Exp. No	Batch	% Initial dry weight	Extract medium	% N in extract	Age of extr. at measurement	pH	Activity of extract			
								In H. U.		In A. U.	
								per ml	per g N	per ml	per g
Arbacia	1	I	0.3	S–w	—	Freshly prepared	6.40	11.4×10^{-9}	—	—	—
»	2	II	0.1	»	—	»	»	54.4 »	—	—	—
»	3	III	0.5	»	0.0012	»	6.60	7.50 »	625.0×10^{-6}	1.90×10^{-5}	158×1
»	4	III	»	»	»	1 day	»	6.31 »	526.0 »	1.30 »	108
Echinus esc.	5	IV	—	—	0.0079	2 1/2 years	6.50	3.33 »	42.2 »	—	—
Arbacia	6	III	0.5	Physiol. saline	0.0014	3 weeks	6.45	9.20 »	657.0 »	1.50 »	107
»	7	III	»	»	0.0017	»	»	1.00 »	59.0 »	—	
»	8	V	»	»	0.0015	Freshly prepared	»	8.10 »	555.0 »	1.30 »	89
Paracentr. ..	9	VI	»	»	0.0020	»	»	6.00 »	306.0 »	1.10 »	56

to the presence of the enzyme found in unfertilized eggs, but the jelly coat activity is much stronger calculated on initial dry weight or nitrogen content in the extract. The activity of a 2.5 per cent extract from unfertilized *Arbacia* eggs is $A = 1.7 \times 10^{-9}$ per ml and of a 0.5 per cent jelly coat solution $A = 11.4 \times 10^{-9}$ per ml. Calculated on a percentage of nitrogen in the extract, which for the jelly coat solution is about 20–30 times less than in the egg extract, the difference is still greater.

DISCUSSION

The experiments give reason to assume the presence of a zymogen—or blocked enzyme—in the unfertilized egg, which is activated at fertilization and which possibly takes part in the processes occurring in the egg after fertilization. After a short period of time (about 30 minutes) the activity is almost abolished by some process *in vivo*. Whether this happens by blocking or by destruction of the enzyme, i.e. whether the process is reversible or

irreversible, is so far impossible to judge. The question whether the egg enzyme and the sperm enzyme are of related or different types will be discussed in the next paper. A noticeable difference is the activity on the alkaline side which, for the egg enzyme, increases but which one for the sperm enzyme decreases from pH 7.7. It seems not improbable that there is more than one enzyme, in the egg, for example one of papain type and another of collagenase type, called prolidase by Smith and Bergmann (13).

SUMMARY

1. A protease effect has been found in sperm extracts from the sea urchin species *Arbacia lixula*, *Paracentrotus lividus* and *Psammechinus miliaris*.
2. Proteolytic activity is found in extracts from lyophilized eggs of *Arbacia* and *Paracentrotus*. Some minutes after fertilization there is a strong increase in activity, followed by a decrease, so that after half an hour the activity is of the magnitude of that in unfertilized eggs or less.
3. Seven hours after fertilization proteolytic activity again appears.
4. Solutions of jelly coats from unfertilized eggs also have a proteolytic effect, tested on gelatin as well as haemoglobin.

The author wishes to thank Professor J. Runnström for his interest and valuable suggestions. He is also indebted to Professor R. Dohrn and Dr. A. Monroy of the Zoological Station at Naples for all their help. His thanks are due to Mrs. A. Runnström for the preparation of the material.

The investigations were financially supported by grants from the Swedish Natural Science Research Council and C. F. Liljevalchs stipendiefond, which is gratefully acknowledged.

REFERENCES

1. Anson, M. L., *J. Gen. Physiol.*, **22**, 79 (1938).
2. Hultin, E., *Svensk Kem. Tid.*, **60**, 40 (1948).
3. Kiermeier, F., *Biochem. Z.*, **318**, 275 (1947–48).
4. Lillie, R. S., *Physiol. Zoöl.*, **14**, 239 (1941).
5. Loeb, J., *Artificial Parthenogenesis and Fertilization.* Univ. Chicago Press, Chicago, 1913.
6. Lundblad, G., *Nature*, **163**, 643 (1949).
7. —— *Acta Chem. Scand.*, **3**, 354 (1949).
8. Northrop, J. H., *J. Gen. Physiol.*, **5**, 353 (1923).
9. —— *ibid.*, **13**, 767 (1930).
10. Runnström, J., *Proc. 6th Intern. Congr. Exptl. Cytol.* (1947).
11. —— *Pubbl. staz. zool. Napoli*, **21**, 9 (1949).
12. —— *The Mechanism of Fertilization in Metazoa*, Advances in Enzymol. **IX**, 241 (1949).
13. Smith, E. L., and Bergmann, Max., *J. Biol. Chem.*, **153**, 627 (1944).
14. Spriggs, E. I., *Z. physiol. Chem.*, **35**, 465 (1902).
15. Vasseur, E., *Acta Chem. Scand.*, **2**, 900 (1948).
16. Woodward, A. A. Jr., *Biol. Bull.*, **97**, 264 (1949).

ON THE ACID FORMATION, BREAKDOWN OF CYTOPLASMIC INCLUSIONS, AND INCREASED VISCOSITY IN PARACENTROTUS EGG HOMOGENATES AFTER THE ADDITION OF CALCIUM

TORE HULTIN

The Wenner-Gren Institute for Experimenta Biology, Stockholm

Received December 15, 1949

If small amounts of calcium ions are added to sea urchin egg homogenates prepared from calcium-free egg suspensions, a series of very rapid processes is released, which is difficult to penetrate by chemical analysis. Because of the important role, which has been attributed to calcium ions not only in fertilization (cf. 21) but also in cell stimulation in general (cf. 6), the study of calcium effects in egg homogenates is of interest as a valuable model procedure.

In previous papers (7, 8) the rapid oxygen uptake was described, which occurs in homogenates of *Psammechinus* and *Paracentrotus* eggs after the addition of calcium. Some evidence was presented in favour of the hypothesis that this oxygen uptake was caused by an unmasking of protein-bound sulfhydryl groups.

ACID FORMATION IN PARACENTROTUS EGG HOMOGENATES AFTER THE ADDITION OF CALCIUM

The acid formation after the addition of calcium to *Psammechinus* egg homogenates was demonstrated in a previous paper (7).

For the study of the acid formation in egg homogenates of *Paracentrotus lividus* (LM.) the eggs were made free from jelly by means of acid treatment. They were washed carefully in citrate-containing Ca-Mg-free artificial sea water (9) 8 times by means of a hand centrifuge. For each Warburg flask 1 ml of centrifuged egg mass was prepared and generally mixed with 0.25 ml of 0.35 M sodium citrate. The

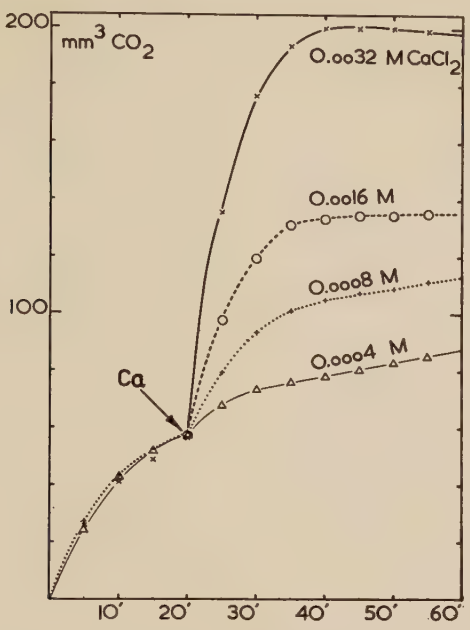


Fig. 1. Influence of different concentrations of $CaCl_2$ on the acid formation of *Paracentrotus* egg homogenate. (Sodium citrate = 0.035 M).

mixture was homogenized by sucking and blowing through a narrow pipette. For each flask 1 ml of isotonic Ca-free Białaszewicz fluid (recalculated from Örström (14) or 0.622 M (9) KCl[1] was added. Of this mixture 2.0 ml were added to each Warburg flask together with 0.1 ml of 0.169 M (9) $NaHCO_3$. In anaerobic experiments a gas mixture of 95 per cent N_2 and 5 per cent CO_2 was conducted through the flasks for 15 minutes. The manometers were closed and the first readings taken 20 minutes after homogenization. The temperature of the thermostate was 18° C. Controls with the reagents added to pure buffered suspension fluid were run.

The acid formation in *Paracentrotus* egg homogenates after the addition of different concentrations of $CaCl_2$ is illustrated by Fig. 1. Full effect was obtained by a total concentration of $CaCl_2$ of about 0.003 M even in the presence of a tenfold excess of sodium citrate. The calcium sensitivity of the homogenates in these experiments was thus about the same as the sensitivity to calcium in the oxygen uptake experiments, described previously (8). These two calcium effects behaved in a parallel manner in another respect also: both declined rapidly between about 1–1 ½ hours

[1] The same results were obtained in both cases. Mg-ions had no significant effect.

after homogenization.[1] These facts indicate a relation between the oxygen uptake and the acid formation in the *Paracentrotus* egg homogenates after the addition of calcium.

The acid formation could be obtained both aerobically and anaerobically. Anaerobically the homogenates generally preserved their calcium sensitivity slightly longer than in the presence of oxygen.

If *Paracentrotus* homogenates were diluted 1: 20 in isotonic KCl and centrifuged for 20 minutes at a rate of 2,000 g, an acid formation after the addition of calcium could be demonstrated by means of a Beckman pH-meter in the slightly opalescent supernatant fluid but not in the washed and resuspended sediment.

Hypertonicity treatment was found (8) to be a useful model procedure imitating the calcium-produced oxygen uptake. However, no acid production was obtained as a result of hypertonicity. If $CaCl_2$ was added 0–35 minutes after hypertonicity a normal acid formation was obtained.

Incubation of homogenates for 35 minutes with monoiodoacetate[2] (0.01 M), $CuSO_4$ (0.001 M), or cystine (sat.) before the addition of calcium reduced the acid production to 20–50 per cent (Fig. 2).

Papain had an effect on the acid formation in *Paracentrotus* homogenates very similar to the calcium effect (Fig. 3).

A concentrated extract of commersial papain (Lemke & Co.) was dialysed against 0.622 M KCl for 24 hours. It was mixed with $\frac{1}{5}$ volume of neutralized 0.1 M KCN in KCl-solution. The pH of the solution was adjusted. In Warburg experiments performed as described above $\frac{1}{10}$ volume of the papain solution was added to the homogenate (pH 6.5). Calcium was added at the same time to homogenate in a parallel flask. Controls were run with boiled papain and KCN added to the homogenate.

After previous papain treatment the acid formation after the addition of calcium was inhibited. As mentioned previously (7) an acid formation in egg homogenates is obtained also after the addition of trypsin. This has been confirmed with dialysed, crystalline trypsin (Armour Lab). The effect is more sluggish than the calcium and papain effects.

---

[1] At 18° C. At higher temperatures the effect was lost more rapidly. In homogenates kept at low temperature (+ 1° C) it could be obtained even after 24 hours (material of 1949).

[2] The addition of iodoacetate or $CuSO_4$ effected *per se* an acidification (Fig. 2). The acid formation after the addition of iodoacetate is explained by reactions suggested in a previous paper (8) to occur during incubation between iodoacetate and sulfhydryl groups in the homogenate (under the formation of free HI). The acidification effected by $CuSO_4$ is probably due, at least partially, to a formation of complexes between Cu^{++} ions and groups in the homogenate. These facts must be remembered if iodoacetate or Cu-salts are used as inhibitors in metabolic studies on sea urchin egg homogenates.

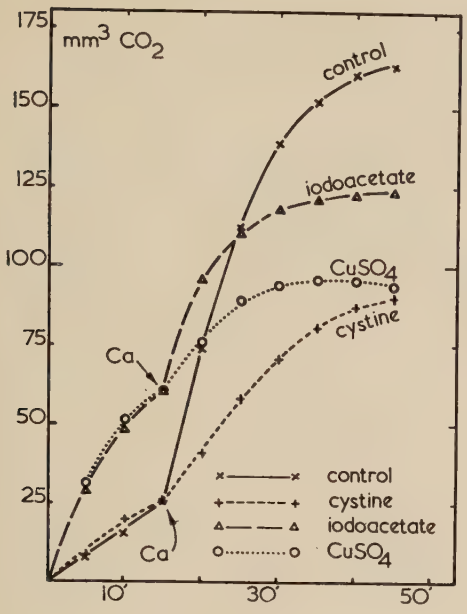


Fig. 2.

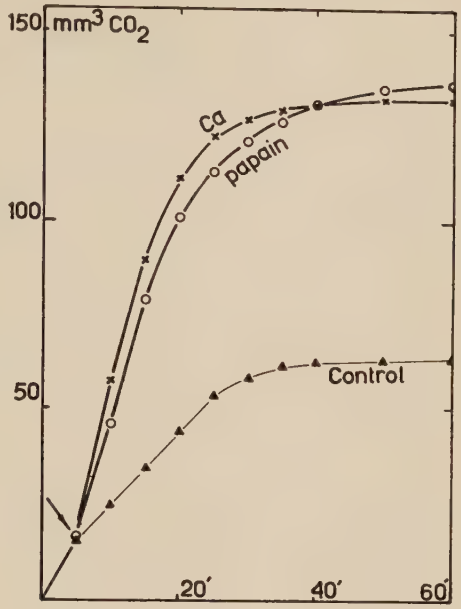


Fig. 3.

Fig. 2. Influence of monoiodoacetate (0.01 M), $CuSO_4$ (0.001 M), and cystine (sat.) on the acid formation of *Paracentrotus* egg homogenate after the addition of $CaCl_2$ (0.003 M). Incubation was started immediately after homogenization. Incubation time 35 minutes (0 minutes = 20 minutes after homogenization). Note the acid formation under the influence of iodoacetate and $CuSO_4$.

Fig. 3. A comparison between the effects of calcium (0.003 M) and papain on the acid formation in *Paracentrotus* egg homogenate. Addition of reagents indicated by the arrow.

COMPARISON WITH THE ACID FORMATION OF WHOLE EGGS IN HYPERTONIC SEA WATER

Runnström (17) observed an acid formation in sea urchin eggs after fertilization. A similar acid formation was found in unfertilized eggs after freeze-thawing (20) or in hypertonic sea water (19). It is very probable (cf. 15) that the permeability of the eggs to calcium ions in the medium is increased during these procedures. If calcium ions from the sea water are allowed to permeate into the eggs their concentration is more than sufficient to initiate full calcium effects.

Jelly-free eggs were carefully washed 6 times in Ca-free artificial sea water. To each Warburg flask were added 2 ml of egg-suspension (ca 8 per cent) by means of a pipette with wide opening. For each experiment 4 flasks were used, 2 aerobic and 2 anaerobic. To one aerobic and one anaerobic flask 0.06 ml of 0.398 M $CaCl_2$

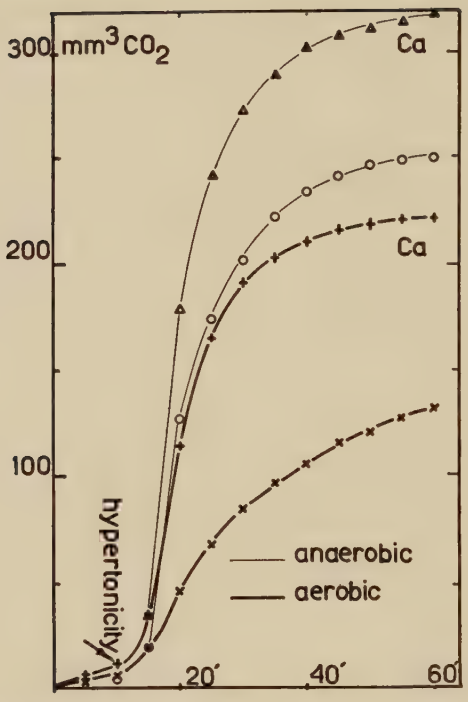


Fig. 4. Acid formation of whole eggs under the influence of hypertonicity in Ca-free and Ca-containing sea water (see text). Hypertonicity = 0.35 ml of 25 per cent NaCl added to 2 ml of egg suspension (19).

had been added corresponding to the calcium concentration of sea water (9). The experiments were then carried out according to the descriptions of Runnström (19). Ten minutes after the first reading 0.35 ml of 25 per cent NaCl were added to all flasks. Cytolysis began after about 5 minutes.

A typical experiment is illustrated by Fig. 4. In the calcium-containing flasks an increased acid formation was obtained, corresponding to the acid formation under the influence of calcium in the homogenate experiments described above. Also in the calcium-free flasks an acid formation was observed. In the homogenate experiments a corresponding acid formation was produced at homogenization. It was still evident after 20 minutes in the beginning of the Warburg experiments (Figs. 1, 2, 3). In agreement with the observation of Runnström (19) the calcium-independent acid formation was higher anaerobically than aerobically (Fig. 4).

The acid formation found by Runnström in whole eggs in sea water under the influence of strong hypertonicity is, thus, made up by at least two components. One component is independent of calcium. It is smaller under aerobic conditions. Another component is produced under the influence of calcium from the medium.

BREAKDOWN OF CYTOPLASMIC INCLUSIONS UNDER THE INFLUENCE OF CALCIUM

When calcium is added to fresh egg homogenates a breakdown of certain cytoplasmic inclusions is observed (cf. Heilbrunn (4)). The details can be studied under the microscope, where it is seen that in *Paracentrotus* homogenates even yolk granules swell and are broken down more or less completely after the addition of calcium. Small vacuoles can often be seen. Macroscopically the effect is very striking as a change in the colour of the homogenate from opalescent, pale red to deep red with much less opalescence. At the same time the viscosity of the homogenate is increased (see below). The breakdown of cytoplasmic inclusions is illustrated by the following experiments.

Equal samples of calcium-free and calcium-treated homogenates were diluted 1: 20 in isotonic KCl and centrifuged for 20 minutes in a cold-room at a rate of 2,000 g. In the calcium-free sample the sediment was red and the supernatant fluid weakly white-opalescent. In the calcium-treated sample the sediment was smaller, and almost white. On the other hand, the supernatant fluid was red-coloured and weakly opalescent.

If the cytoplasmic inclusions were isolated from dilute (1: 20) homogenate by means of a centrifuge, and thereafter resuspended in isotonic, calcium-free Białaszewicz fluid or KCl-solution, they were no longer influenced by added $CaCl_2$. Even if homogenates were diluted, the reaction became more and more incomplete. In fresh homogenates, therefore, a factor is supposed to be present in the cell fluid, capable of breaking down cytoplasmic inclusions after activation by calcium. Trypsin, pancreatic lipase or lysolecithine had no visible influence on the granules. The only substance, so far investigated, with a macroscopically evident effect was papain. The yolk granules, however, were not dissolved by papain.

If *Paracentrotus* homogenates (1 : 1) were treated for 30–40 minutes with iodoacetate (0.01 M), $CuSO_4$ (0.001 M), or cystin (sat.), the breakdown of granules under the influence of calcium could often be more or less inhibited. If the inhibition was incomplete, the yolk granules were swollen but not dissolved.

The breakdown of cytoplasmic inclusions could only be obtained in fresh homogenates. The decline of the effect was parallel to that of the acid formation and oxygen uptake (8) described previously (generally 1–1 ½ hours after the homogenization, at 18° C, in the 1949 material).

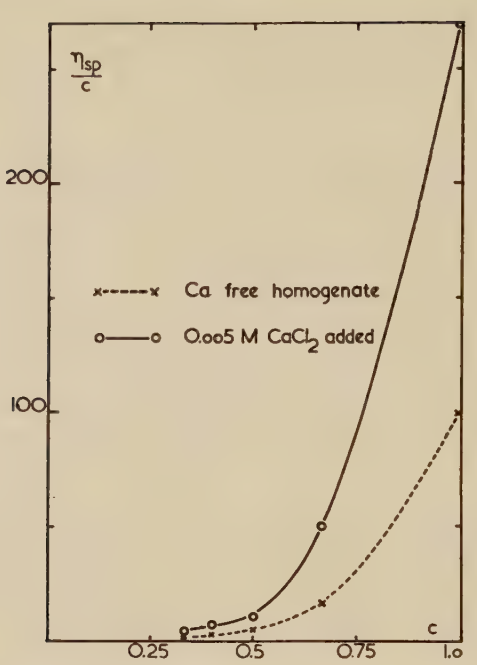


Fig. 5. The effect of $CaCl_2$ (0.005 M) on the viscosity of *Paracentrotus* homogenates of different concentrations. Concentrations (c) of homogenates expressed as fractions of concentrated (c = 1) homogenate. η_{sp} = spec. viscosity.

No breakdown of granules was obtained by hypertonicity. In hypertonic homogenates the yolk-granules stuck together and the whole suspension became white-flocculent (7). Such clusters of granules could no longer be dissolved by subsequent addition of calcium (cf. 5).

THE INCREASED VISCOSITY OF EGG HOMOGENATES AFTER THE ADDITION OF CALCIUM

Paracentrotus eggs were homogenized as described above and diluted with isotonic Białaszewicz solution in the following proportions: 1+0, 1 + ¼, 1 + ½, 1 + ¾, 1+1 etc. The viscosity before and after the addition of calcium was determined in Ostwald viscosimeters. A small dilution was caused by the addition of calcium (0.063 ml of 0.398 M $CaCl_2$ to 5 ml of homogenate). Temperature 18° C.

The viscosity of homogenates of different dilutions and the effect of calcium on the viscosity is illustrated by Fig. 5, which represents a typical

TABLE I

Viscosity effect in aging homogenates.
η_{sp} = spec. viscosity: c = homogenate concentration expressed as fraction of concentrated (c = 1) homogenate.

8/3 1949	c = 0.445		10/3 1949	c = 0.57	
Minutes after homogenization	η_{sp}		Minutes after homogenization	η_{sp}	
	Before Ca addition	After Ca addition		Before Ca addition	After Ca addition
30	1.47	3.03	40	5.47	18.7
40	1.68	3.14	50	6.40	17.3
50	1.75	2.50	60	7.00	8.78[1]
70	2.02	1.63	70	6.27	4.03

TABLE II

Influence of iodoacetate (0.01 M) and $CuSO_4$ (0.001 M) on the viscosity effect.
η_{sp} = spec. viscosity; c = homogenate concentration expressed as fraction of concentrated (c = 1) homogenate.

21/3 1949	c = 0.5		26/3 1949	c = 0.445	
η_{sp}	Before Ca addition	After Ca addition	η_{sp}	Before Ca addition	After Ca addition
Iodoacetate 35 min.	5.47	4.87	$CuSO_4$ 30 min.	2.82	2.69
Control 27 min.....	3.43	7.08	Control 35 min.....	1.6	4.89
Iodoacetate 45 min.	4.37	3.50			

experiment. After the addition of calcium an increased viscosity was obtained especially in the concentrated homogenates. Under the influence of calcium concentrated homogenates became sticky and could to some extent be pulled out to elastic strands by means of a glass rod. The viscosity of the homogenates was very sensitive to dilution. In the Fig. 5, and in the following tables the homogenate concentrations (c) are expressed as fractions of concentrated (c = 1) homogenate.

Like the other calcium effects the increased viscosity could also be produced only in fresh homogenates. At 18° C the effect could not be obtained longer than 1–1 ½ hours after homogenization (Table I). Before the end of the calcium-sensitive period a spontaneous increase in viscosity was generally observed (cf. 8).

Incubation of homogenates for 30–40 minutes with iodoacetate (0.01 M)

[1] Incomplete breakdown of cytoplasmic inclusions.

Table III

Influence of papain on the viscosity of homogenates.
η_{sp} = spec. viscosity; c = homogenate concentration expressed as fraction of concentrated (c = 1) homogenate.

21/4 1949	c = 0.445	
η_{sp}	Before Ca addition	After Ca addition
Control	1.33	3.71
Papain-homogenate ...	2.70	2.00

or $CuSO_4$ (0.001 M) was in most cases sufficient to prevent the viscosity effect after the addition of calcium. Iodoacetate or $CuSO_4$ themselves caused a certain increase in the viscosity of the homogenates (Table II). This effect was probably due to an oxidation of protein-bound sulfhydryl groups by these reagents (cf. 8).

Papain increased the viscosity of *Paracentrotus* homogenates but to a lesser degree than calcium. Calcium added 15 minutes after the addition of papain had no effect (Table III). Trypsin (Armour Lab., cryst.) had no effect on the viscosity.

A concentrated extract of papain (Lemke & Co.) in distilled water (5 ml) was dialysed for 24 hours against Ca-free Białaszewicz fluid, and 1 ml of neutralized, 0.1 M KCN was added. The pH of the solution was adjusted to 6.5. To each ml of diluted (1: 1) homogenate 0.25 ml of this solution was added. The same amount of heated and centrifuged papain solution was added to the control.

Mirsky (12) found a decrease in the solubility in M KCl of the proteins in the *Arbacia punctulata* eggs after fertilization. Experiments were performed to investigate if in the homogenates the increased viscosity after the addition of calcium was accompanied by a diminished solubility of the proteins.

In these experiments egg homogenates of both *Paracentrotus lividus* and *Sphaerechinus granularis* (LM.) were used. The concentrated egg homogenates were divided into 6 equal samples. $CaCl_2$ (0.005 M) was added to 3 of these samples. The samples were diluted with 20 volumes of respectively (a) distilled water, (b) isotonic KCl, and (c) M KCl. They were centrifuged for 20 minutes at a rate of 2,000 g. In the calcium-treated *Paracentrotus* homogenates the red sediment of cytoplasmic inclusions had disappeared more or less completely. Instead, the supernatants were pale red. The supernatants were precipitated by addition of trichloroacetic acid (10 per cent) and the nitrogen contents of the washed precipitates were determined.

TABLE IV

Influence of calcium on the amounts of homogenate proteins soluble in different media.

Homogenates diluted by	Ca-free homogenate	Ca-treated homogenate
(a) dist. water	1.05 mg N	1.25 mg N
(b) 0.622 M KCl	0.90	1.15
(c) M KCl	0.85	1.00

The result of one typical experiment is given in Table 4. It is evident that the eventual decrease in the solubility of the proteins is widely overcompensated by the release of trichloroacetic acid insoluble material from the broken down cytoplasmic inclusions.

DISCUSSION

It was shown in a previous paper (8) that calcium ions have a slightly inhibiting effect on the respiration and phosphorylation of egg homogenates. The acid formation obtained after the addition of calcium is therefore not supposed to be due to carbohydrate metabolites.

The specific and very high affinity of sea urchin egg homogenates for calcium ions was demonstrated previously (8). This affinity is higher than the affinity of calcium for citrate. The acidification described in the present study can be due, at least partially, to the incorporation of calcium ions into very slightly dissociated complexes with acid groups in the homogenate (cf. 3). It is not sure, however, that the whole effect is of such physicochemical nature.

The close similarity between the calcium effect and the acid formation by papain (Fig. 3) proves that such effects can be caused by proteolytic enzymes. Proteolytic activity has been found in sea urchin eggs (10). It is temporarily increased during fertilization. It was evidenced above that cytoplasmic inclusions are broken down under the influence of factors in the cell fluid, which are activated by calcium. The capacity of the homogenates both to break down cytoplasmic granules and to produce acid, under the influence of calcium, is lost under the same conditions. Both effects are therefore supposed to be released over the same mechanism. This

mechanism is tentatively thought to be of enzymatic[1] (proteolytic[2]?) nature and brought into activity by calcium. The high ionic specificity of the calcium effects and their sensitivity to incubation with sulfhydryl reagents is in agreement with this hypothesis (2, 16). Most likely, certain protein-complexes containing mucous or lipid components are broken down under the influence of a calcium-activated factor.

The increased viscosity in the homogenates after the addition of calcium is probably of complex origin. A direct precipitation of colloid elements in the homogenate by the calcium ions is not supposed to be the only cause of the increased because of the rapid disappearance of the calcium effect in aging homogenates. Material liberated from the broken down cytoplasmic inclusions contributes to the effect. Most likely, a sulfhydryl mechanism (8) is involved (cf. 13). The possibility of a calcium-activated clotting system in the homogenate should not be excluded. Already Heilbrunn (4) emphasized the analogy between the "surface precipitation reaction" and blood coagulation. The increased viscosity under the influence of papain is in agreement with this idea (1).

SUMMARY

1. In fresh *Paracentrotus* egg homogenates a rapid acid formation was found after the addition of small concentrations of $CaCl_2$. At the same time certain cytoplasmic inclusions swelled and were more or less completely broken down. The viscosity of the homogenates increased 100–200 per cent.

2. The acid formation was most likely due not only to an incorporation of Ca^{++} ions into complexes with acid groups in the homogenate. It was not supposed to be caused by carbohydrate metabolites. An acid formation very similar to the calcium-produced acid formation was caused by papain. A breakdown of certain cytoplasmic granules, and an increased viscosity was observed also under the influence of papain. The calcium effects mentioned above could not be superimposed on the papain effects.

3. Isolated cytoplasmic granules, resuspended in isotonic, Ca-free Białaszewicz solution of KCl, were no longer influenced by calcium. Calcium is therefore supposed to act through a factor in the cell fluid. As a working hypothesis, this factor is considered to be identical with the acid-producing factor and possibly of enzymatic nature.

---

[1] Cf. Heilbrunn (5).

[2] The effect of different concentrations of $CaCl_2$ on the acid formation of homogenate (Fig. 1) is in certain respects similar to the activating effect of different concentrations of Ca on the formation of trypsin from trypsinogen (11). The reaction in the homogenates is more rapid and more sensitive to Ca.

4. The acid formation under the influence of calcium is one constituent of the complex acid formation, which was found by Runnström (19) to occur in whole sea urchin eggs in hypertonic sea water.

ACKNOWLEDGEMENTS

The investigation was carried out at the Zoological Station of Naples. The author wishes to express his gratitude to the Station Authorities for laboratory facilities and material. He wishes to express his great indeptedness to Professor J. Runnström for all help and interest, and to Fil. Mag. Karin Hultin for valuable assistance. The investigations were supported by grants awarded to the Wenner-Gren Institute by the Swedish Natural Science Research Council.

REFERENCES

1. Eagle, H., and Harris, N. T., *J. Gen. Physiol.*, **20**, 543 (1937).
2. Fruton, J. S., Irving, G. W., and Bergmann, M., *J. Biol. Chem.*, **138**, 249 (1941).
3. Greenwald, I., *J. Biol. Chem.*, **124**, 437 (1938).
4. Heilbrunn, L. V., *Arch. Exp. Zellforsch.*, **4**, 246 (1927).
5. —— *Protoplasma*, **11**, 558 (1930).
6. —— *An Outline of Gen. Physiol.*, London (1937).
7. Hultin, T., *Arkiv Kemi, Mineral. Geol.*, **26** *A*, No. 27 (1949).
8. —— *Exper. Cell. Research*, **2**, 159 (1950).
9. Hörstadius, S., *Pubbl. Staz. Zool. Napoli*, **14**, 251 (1935).
10. Lundblad, G., *Nature*, **163**, 643 (1949).
11. McDonald, M. R., and Kunitz, M.. *J. Gen. Physiol.*, **25**, 53 (1941–42).
12. Mirsky, A., *Science*, **84**, 333 (1936).
13. Monroy, A., and Runnström, J., *Arkiv Zool.*, **40** *A*, No. 18 (1948).
14. Örström, Å., *Z. Physiol. Chem.*, **271**, 1 (1941).
15. Pasteels, J., *Bull. Acad. R. Belg.*, **24**, 721 (1938).
16. Purr, A., *Biochem. J.*, **29**, 5, 13 (1935).
17. Runnström, J., *Arkiv Zool.*, **21** *B*, No. 8 (1930).
18. —— *Protoplasma*, **15**, 532 (1932).
19. —— *Biochem. Z.*, **258**, 257 (1933).
20. —— *Biol. Bull.*, **69**, 345 (1935).
21. —— *Adv. Enzymology*, **9**, 241 (1949).

ON THE EFFECT OF ADENOSINE TRIPHOSPHORIC ACID AND OF CA ON THE CYTOPLASM OF THE EGG OF THE SEA URCHIN, PSAMMECHINUS MILIARIS

JOHN RUNNSTRÖM and GEORG KRISZAT

The Wenner-Gren Institute for Experimental Biology, Stockholm

Received January 6th, 1950

SURVEY OF THE PROBLEM

The role of adenosine triphoshoric acid (ATP) in the intermediate metabolism has been the subject of a vast literature. It is now recognized that ATP acts as an accumulator of energy which may be made available for different processes in the cell. The mechanism of such a transfer is still obscure. The studies of Szent-Györgyi (26, 27), Buchthal (1) and others throw, however, a certain light on the interaction of ATP with the contractile substance of the muscle fibre. Electron microscopic studies (19) suggest that ATP is able to split actomyosin into its components, actin and myosin. After the splitting a precipitation of the components occurs if the dispersion medium is 0.05 N KCl.

The sea urchin egg is rather rich in an easily hydrolyzable phosphate ester which no doubt mainly corresponds to ATP; see (23, p. 307). Likewise is the egg rich in ATP-ase, the activity of which possibly increases upon fertilization; see (23, p. 310).

Runnström (22) observed that treatment of Psammechinus eggs with 0.005 M ATP impedes the formation of a normal fertilization membrane. This membrane becomes low with an irregular shape. In work with eggs of *Arbacia lixula,* Wicklund found that eggs kept in sea water often lose their capacity of forming a normal membrane upon fertilization. Addition of ATP to a concentration less than 0.001 M protects the egg against this change; see (23, p. 251). Wicklund also found that the properties of the cytoplasm and the vitelline membrane change radically upon exposure to ATP 0.001–0.002 M. The ATP-treated eggs proved to be more resistant to hypotony

than the control eggs; see (23, p. 285). ATP thus seems to exert a striking effect on the structure of the cytoplasm of the sea urchin egg.

In view of these considerations, the present writers were interested in examining the general effect of ATP on the cytoplasm of the sea urchin egg. For this purpose the eggs were centrifuged, and stratification of the cytoplasm was observed. It is well-known that particularly Heilbrunn (6–8) and his co-workers have utilized the centrifuge method to obtain relative or even absolute values of the viscosity of the cytoplasm. In the present work, the method of suspending the eggs on the border between sea water and isotonic sucrose solution was also used. This method was introduced by E. N. Harvey and Loomis (5) and has been utilized in numerous experiments by E. B. Harvey (see for references, 23, p. 289).

As an introduction to the planned work, one of the writers (12) undertook experiments on the Amoeba, *Chaos Chaos*. Exposure of the amoebas to ATP gave rise to an initial decrease followed by an increase of apparent viscosity.

ATP added to the culture medium of amoebae or to sea water is slightly precipitated by Ca present in the media. Attempts were therefore made to work in media deprived of Ca. In Ca-free medium, however, the rate of stratification decreases considerably. This has already been demonstrated to be the case with animal as well as with plant cells, particularly by Heilbrunn (7) with eggs of *Arbacia punctulata*, and by Heilbrunn and Daugherty (8) with *Amoeba dubia*.

The use of Ca-free medium thus introduces a complication. Later it was found that ATP in normal sea water has a pronounced effect. The solubility product of the Ca salt is thus large enough to cause an effect on the egg.

Changes in the structure of cytoplasm were also studied by observing eggs transferred to a strongly hypotonic medium. Eggs degraded by hypotony following pretreatment with Ca-free sea water proved to be particularly useful for studying the effects of ATP and Ca.

MATERIAL AND METHODS

The material used was the litoral form of *Psammechinus miliaris*. The eggs were generally kept in sea water with a salinity of 25 per mille. Ca-free sea water was prepared according to the table published by Öhman (18) and was then diluted with redistilled water to 25 per mille. The pH of the

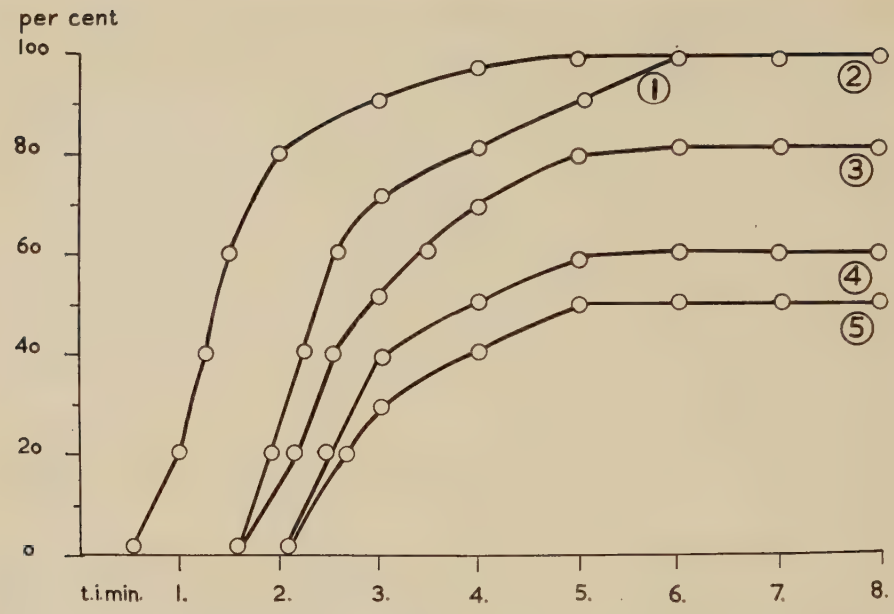


Fig. 1. Curves giving stratification as a function of time. Zero time is the moment when the speed had reached a constant value. The media were: 1. Sea water. 2. Sea water with twofold increase of the Ca content. 3. Sea water with 0.003 M adenosine triphosphate 4. Ca-free sea water with 0.003 M adenosine triphosphate (*Psammechinus miliaris*).

artificial mixtures was adjusted to pH 8.1–8.2. Sea water with increased Ca-content was obtained by adding 3.7 per cent solution of $CaCl_2$ to sea water.

A Ba-salt of ATP of 80 per cent purity was prepared for us by Mr. L. Ernster.

The Ba-salt was converted into Na_4 ATP. A 0.1 M solution of this salt was then added to the Ca-free or Ca-containing sea water to obtain a 0.003 M concentration of ATP.[1] This was the concentration generally used throughout the experiments. The eggs were always incubated for one hour with ATP-solution. The temperature was 20°–22° C.

The centrifuge microscope used was manufactured by the firm Struers and Co., Copenhagen.

Ten or twenty of the pretreated eggs were placed in a microcuvette. Control eggs were placed at the same time in a parallel cuvette. The same time

[1] Owing to the precipitation following addition of 0.003 M ATP to sea water the Ca-concentration decreased with about 6 per cent, the amount of added organic P with about 1 per cent.

and rate of centrifuging was maintained throughout the experiments. In the course of some minutes from the start the speed reached a constant value, corresponding to the acceleration 4,350 g. The zero point on the curves in Fig. 1 indicates the moment when this speed was attained. The centrifuge was then kept running for 8 more minutes before the motor was switched off. The time between zero and the beginning of stratification is referred to below as the "delay."

The displacement of yolk granules and of mitochondria was observed during the centrifuging. The results were judged also from the conditions prevailing immediately after the centrifuging. The cuvettes were now removed from the centrifuge and photographs were taken with a Leica camera mounted on an ordinary microscope.

When the procedure adopted by E. N. Harvey was adhered to, the microcuvettes were filled to $\frac{2}{3}$ with 23 per cent sucrose solution. Above the sugar solution came a layer of normal or modified sea water to which the eggs had been exposed during pretreatment. The time and speed of centrifuging were the same as mentioned above. At the centrifugal force adopted in our experiments, in general, no fragmentation of the egg occurred. Other methods used will be described below.

RESULTS OF THE CENTRIFUGE EXPERIMENTS

The stratification obtained in control eggs is shown in Fig. 2 a (centrifugal direction downwards). The mitochondria accumulate at the heavier egg pole. The next layer separated off is devoid of coarser inclusions. It is formed by the ground cytoplasm which in this species is heavier than the yolk granules as was earlier found by Lindahl (13) and Monné (17). An oilcap rarely appears in normal sea water but becomes visible when the rate of stratification of cytoplasm is increased by addition of Ca to sea water. After 8 minutes centrifugation the yolk material is limited to the centripetal half of the egg separated from the layer of mitochondria by the hyaline band. The conditions represented in Fig. 2 a are designated as 100 per cent stratification. Fig. 2 b shows the stratification in sea water + ATP, 2 c in Ca-free sea water and 2 d in Ca-free sea water + ATP.

The time course of the separation has been plotted in the curves of Fig. 1. The points give the stratification in per cent of the final stratification in the control eggs at different times. Each value is the average of 10 determinations.

In control eggs the delay (see above) is about 1.6 minutes. After 6

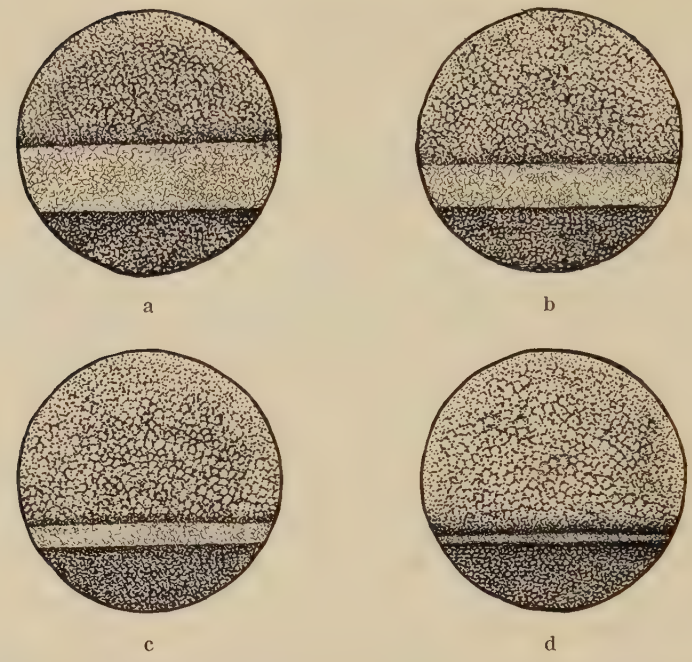


Fig. 2. Stratification of the eggs. a. In sea water. b. In sea water with 0.003 M adenosine triphosphate. c. In Ca-free sea water. d. In Ca-free sea water with 0.003 M adenosine triphosphate (*Psammechinus miliaris*).

minutes 100 per cent stratification is reached; see Fig. 1, curve 1. After exposing the eggs to sea water with a twofold increase of the Ca-content, the delay is much shorter, viz. about 0.5 minutes; 100 per cent stratification is reached after 4 minutes, see Fig. 1, curve 2. In the Ca-free medium (Fig. 1, curves 4 and 5) the delay is prolonged to 2 minutes. When ATP is added to Ca-containing sea water or, when the eggs are immersed in Ca-free sea water or Ca-free sea water + ATP (Fig. 1, curves 3–5) 100 per cent stratification is not attained with the force applied. The final stratification is 80, 60 and 50 per cent, respectively.

The curves of Fig. 1 are sufficient to show that the egg cytoplasm is not a viscous fluid, see also (2). All the curves should then begin at the same point and their asymptotes should be on the same level. The centrifugal force which tends to stratify the eggs is opposed by the presence of an interior fabric, the rigidity of which is dependent on bonds of different strengths which keep the units of the fabric together. The bonds are broken by the impact of granules. The delay is accounted for by the strong resistance of certain

bonds. Let us assume that the impact is exerted by granules of a diameter of 1–2 μ and that they act at a distance of 1 Å. The energy of the bonds which are broken is then calculated to be of the order 1–10 Cal. These values correspond to the energy of hydrogen bonds or of cohesion bonds between organic groups; see (3, p. 24).

When the process of stratification has once started, it goes on at a rather high rate. The maximum slopes of the different curves were the following:

	Curve				
	1	2	3	4	5
Maximum slope (per cent stratification per minute)....	61.5	78	39	40	27

The maximum slopes decrease with the decrease of Ca content. Furthermore, addition of ATP decreases the slopes considerably, though the delay is not greater than in the corresponding controls. From the above it must be inferred that ATP reinforces the bonds which keep the fabric of egg cytoplasm together. The resistance offered by the bonds, is strong enough to obstruct the attainment of a stratification to the same extent as found in the control. The strength of the binding forces is apparently also highly dependent on the Ca-content of the medium. They decrease with increasing Ca-concentration.

Experiments carried out with eggs suspended in the border region between sea water and sucrose solution showed on the whole the same result as those recorded above. Fig. 3, I–V, indicates diagrammatically the changes observed during the centrifugation of the eggs. The uppermost horizontal row represents the conditions observed after switching off the motor. In this stage the stratification is almost exactly the same as in eggs centrifuged in a uniform saline medium.

The first vertical row, I in Fig. 3, shows the behavior of control eggs in normal sea water. They alongate and after a certain time assume a dumb bell shape (I, 3). The centrifugal force adopted was not sufficient to bring about the strong elongation and separation into fragments so well described and illustrated by E. B. Harvey (4). The mitochondria layer appeared early in the centrifugal part of the egg, but beyond this layer a hyaline zone was present. When the motor was switched off this hyaline zone disappeared. It was probably incorporated into the adjacent more centripetal layers. In the eggs subjected to sea water with increased Ca-content (II) the hyaline zone between the mitochondria and yolk layers becomes broader, and the whole egg elongates more than that in the control. A centrifugal hyaline cap persists even after switching off the motor. The eggs subjected to Ca-

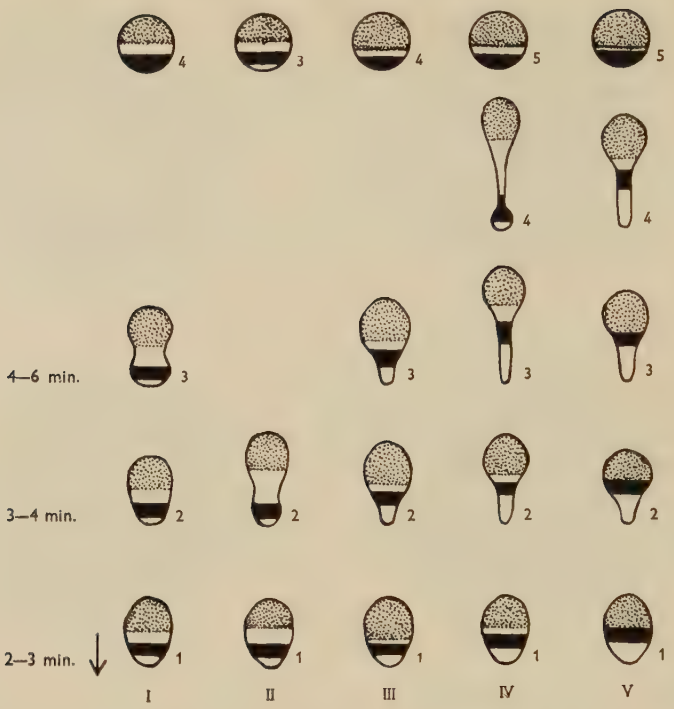


Fig. 3. Diagram to show the effect of centrifuging eggs suspended in the border between sea water and sucrose solution. The media were: I. Sea water. II. Sea water with twofold increase of the Ca content. III. Ca-free sea water. IV. Sea water with 0.003 M adenosine triphosphate. V. Ca-free sea water with 0.003 M adenosine triphosphate.

free sea water (III) behave rather differently. The hyaline zone enclosed between the mitochondria and the yolk layer never becomes as broad as in the preceding cases. The centrifugal hyaline zone becomes here cone-shaped, a feature which is still more conspicuous in eggs treated with ATP (IV and V).

The elongation visible in IV may in certain cases result in separation of the egg in one heavy, small and one light, big fragment. The centrifugal hyaline zones must be constituted by material that is heavier than mitochondria. Their appearance is no doubt due to the contact of the centrifugal part of the eggs with the sucrose solution.

Fig. 4 and Fig. 5 represent eggs centrifuged on the border between sucrose solution and Ca-free sea water, respectively without (Fig. 4) and with (Fig. 5) addition of ATP. The texture of cytoplasm is obviously coarser and more refractive in the former than in the latter. Fig. 6 represents an egg centri-

fuged on the border between sucrose solution and sea water with a Ca concentration twice the normal. The two hyaline zones described above are visible on the figure. The texture of cytoplasm seems also in this case to be finer than that in the Ca-free medium.

The rate of redistribution of materials after centrifugation was greater in the eggs showing a high degree of stratification than in those with less pronounced stratification.

THE BEHAVIOUR OF EGGS UPON DILUTION OF SEA WATER OR CA-FREE SEA WATER WITH DISTILLED WATER

Valuable information can be gained when observing eggs which have cytolyzed subsequent to strong dilution of the medium with distilled water. The eggs were kept in dishes with about 2 ml normal or Ca-free sea water. About 12 ml redistilled water was added and the eggs sank to the bottom. The water was then removed by means of a fine pipette, connected with a water pump, until the volume was reduced to about 2 ml. Eggs of *Echinocardium cordatum* were generally used for these experiments, but the results proved to be valid in all essential respects also for *Psammechinus* eggs.

The eggs pretreated with Ca-free sea water swelled rapidly and became brownish. The shape of the eggs was spherical. Each egg contained a fine homogeneous framework which extended to the surface. The outline of the last named was not strongly marked. The vitelline membrane was not visible or at most demonstrable as a very delicate structure, see Fig. 7.

The eggs from normal sea water behaved rather differently. When they were transferred to the diluted medium, they swelled more and did not present the homogeneous spherical appearance described for the eggs from the Ca-free sea water. The cytoplasm was richer in vacuoles, which developed particularly below the surface layer. The framework was considerably coarser and less homogeneous than in the eggs pretreated with Ca-free sea water, compare Figs. 7 and 8. The vitelline membrane was more readily visible. It often grew thicker, but in general not uniformly over the whole surface. In an optical section local thickenings could be observed. The swelling of the eggs was stronger in a Ca-containing than in a Ca-free medium, as the following data show. Eggs from normal sea water (I) and from Ca-free sea water (II) were strongly diluted, as described above. After 10–15 minutes, the eggs were measured by means of a screw micrometer and the following values were obtained:

$$\begin{aligned} &\text{I } 143.1 \pm 1.61 \qquad (n = 21)\\ &\text{II } 132.2 \pm 1.39 \qquad (n = 20)\end{aligned}$$

$$D = I - II = 10.9 \pm 2.13, \frac{D}{e} = 5.15$$

D is the difference, e the standard deviation of the difference, which is highly significant. The numbers indicate scale units on the micrometer, each unit = 1.1 μ.

The eggs transferred from normal sea water often burst at one point. From this point part of the framework; and particularly hyaline blisters were expressed (Fig. 8).

Gradually an increasing number of blisters left the eggs and were scattered in the medium; see Fig. 9. This kind of blister was considered to be of lipid nature (11, 20). Under favourable conditions, a birefringence, negative with respect to the radial directions was observed. When 2.5 per cent phosphotungstic acid was added the outline of the blisters became sharper and the birefringence changed its sign. This indicates that the blisters are probably composed both of proteins and lipids.

In sharp contrast to the plentiful blister formation in eggs pretreated with normal sea water, no blisters appeared when eggs pretreated with Ca-free sea water were immersed in the diluted medium. This was perhaps the most striking difference between the two kinds of eggs.

Single blisters were yet formed if the eggs were pretreated for 8–10 hours in Ca-free sea water before adding distilled water, see Fig. 10. These blisters were pigmented and much denser than in eggs pretreated with normal sea water. They remained attached to the egg surface by means of a stalk.

In the interior of the staled eggs, pretreated with Ca-free sea water, rather dense myeline figures were visible. These are the precursors of blisters. It appears from Fig. 10 that the framework of the cytoplasm retained its rather homogeneous appearance also in staled eggs.

After 10–12 hours' exposure of the eggs from Ca-free medium to distilled water the blisters were still stalked. They contained, however, a number of densely aggregated blisters, enclosing in their turn smaller blisters. The complicated pattern thus arising was observed also in swelling lecithin "ex ovo" (Merck, Darmstadt), see (20).

When *Psammechinus* eggs pretreated with normal sea water were transferred to a strongly hypotonic medium, the vitelline membrane burst at one point. The outflowing cytoplasm thus protruded from a bag-like thin vitelline membrane, see Fig. 14. After 5–10 minutes, hyaline blisters appeared

and were scattered in the medium. After some hours the cytoplasmic framework finally began to separate into blisters with granular walls. The interior of the blisters also contained granules in Brownian movement.

In eggs pretreated with Ca-free water and then transferred to strongly hypotonic medium, the surface burst in one or two points, but in general the outflow of cytoplasm was limited. The cortex did not detach itself from the vitelline membrane. The interior consisted of a homogeneous framework which contrasted with the coarser and more irregular framework of the egg pretreated with normal sea water.

Sea urchin eggs remain fertilizable for a longer time in Ca-free than in normal sea water (14, 25). In accordance with this the mature unfertilized eggs cytolyze earlier in normal than in Ca-free sea water. Figs. 11 and 12 show the eggs after exposure for about 24 hours to the respective media diluted with equal parts of isotonic sucrose solution. Almost all the eggs in normal sea water + sucrose were cytolyzed. Entire eggs or, more generally, certain regions of the eggs were subdivided into numerous irregular fragments. After the same time period, very few of the eggs kept in Ca-free sea water + sucrose had cytolyzed. They were spherical and their surface was very weakly refractive.

THE INFLUENCE OF ATP ON THE BEHAVIOUR OF THE EGGS UPON DILUTION OF THE MEDIUM

Unfertilized *Psammechinus* eggs were pretreated for 60 minutes with 0.003 M ATP in sea water. The medium was then highly diluted in the manner described in the preceding section. The vitelline membrane proved to be more resistant than in a parallel control which was also subjected to the diluted medium.

The eggs pretreated with ATP-sea water remained almost spherical. The ground substance of cytoplasm appeared as a coherent framework. The bulk of cytoplasm was still covered by the vitelline membrane. The latter was much more refractive than following pretreatment with normal sea water. A precipitation of the vitelline membrane had evidently occurred.

Exovates were formed in many cases, but they were comparatively small and remained fixed to the bulk of cytoplasm often by means of a stalk. No blisters were detached from the exovates.

The difference between eggs pretreated with normal and with ATP-sea water is illustraded in Figs. 14 and 15.

Sometimes the membrane was granular, demonstrating that the cortical granules had united with the vitelline membrane as a consequence of the ATP action. The behaviour of the eggs in a diluted medium strongly indicates that the submicroscopic constituents are more intimately linked following pretreatment with ATP than in the eggs pretreated with normal sea water. Our results agree with those obtained earlier by Wicklund in work on other species (23, p. 285).

Eggs of *Echinocardium* were transferred to Ca-free sea water and kept there for two hours. Small quantities of eggs were then immersed in distilled water. The eggs broke up in the typical manner described above.

After 5 minutes the following additions were made to 2 ml distilled water:

1 none
2 0.1 ml 0.35 $CaCl_2$
3 0.3 ml 0.01 M ATP
4 0.3 ml 0.01 M ATP + 0.1 ml 0.35 M $CaCl_2$

In 1 no changes occurred and none or very few dense blisters appeared.

In 2 the typical phenomena occurred as described above. The eggs appeared more brownish and numerous blisters were formed which escaped mainly through the micropyle. A rather irregular thickening of the vitelline membrane occurred. Generally the thickened parts were localized to separate parts of the surface.

In 3 numerous blisters appeared, but the surface remained unchanged until 0.1 ml 0.35 M $CaCl_2$ was added. The vitelline membrane then coagulated. In many cases the cytoplasm retracted from the membrane, but remained connected with the latter through numerous threads or strands.

Changes similar to those just described occurred immediately in 4. Fig. 16 shows a membrane which occupied a great part of the egg. It was separated from the egg surface by a vitelline space. However, numerous strands were present. An egg with a more irregularly formed membrane is represented in Fig. 17. It was formed by a number of local thickened patches which did not separate from the egg surface. In some eggs the membrane thickening occurred only in rather few isolated patches. Also the interior cytoplasm was different in 3 and 4 on one side, and 1 and 2 on the other. Compare Figs. 16 and 17 with Figs. 7 and 8.

The central cytoplasm has a much finer texture in 3 and 4 than in 1 and 2. These observations tend to show that ATP has a definite influence on the cytoplasm of eggs broken up in Ca-free sea water. This action is reinforced upon addition of Ca.

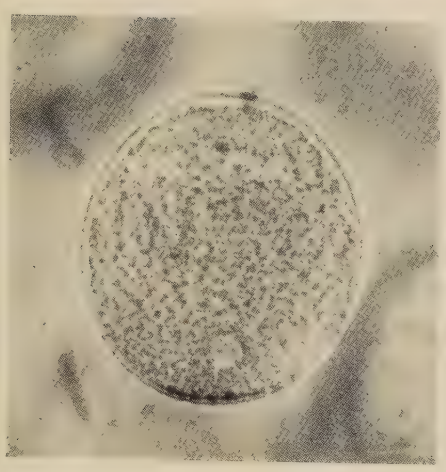


Fig. 4.

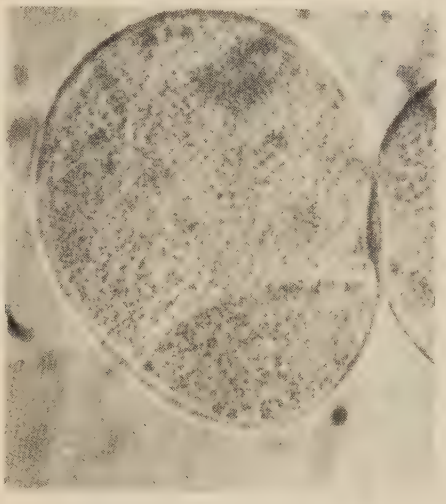


Fig. 5.

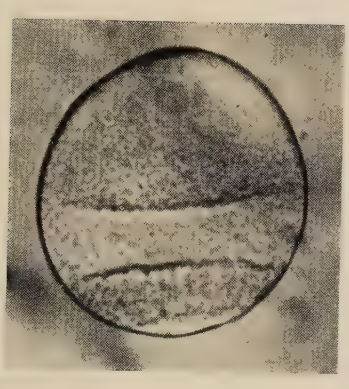


Fig. 6.

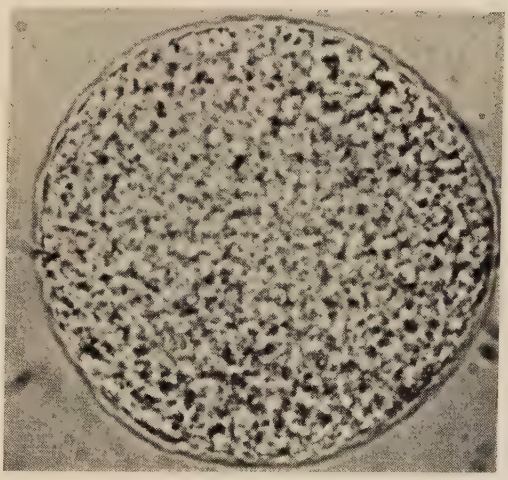


Fig. 7.

Fig. 4. Egg centrifuged in the border between Ca-free sea water and sucrose solution.
Fig. 5. Egg centrifuged in the border between Ca-free sea water with 0.003 M adenosine triphosphate and sucrose solution.
Fig. 6. Egg centrifuged in the border between sea water with twofold increase of the Ca-content sucrose solution.
Fig. 7. Egg pretreated in Ca-free sea water immersed in strongly diluted medium (*Echinocardium cordatum*).

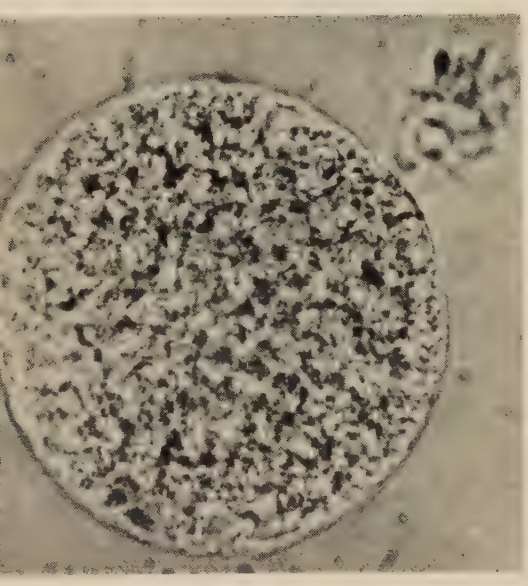


Fig. 8.

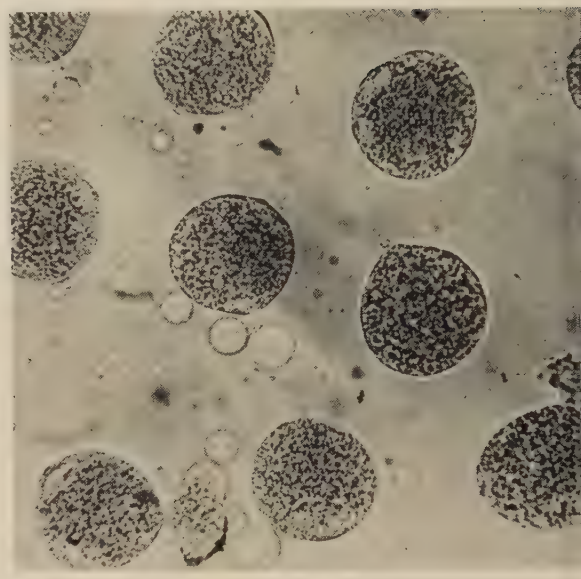


Fig. 9.

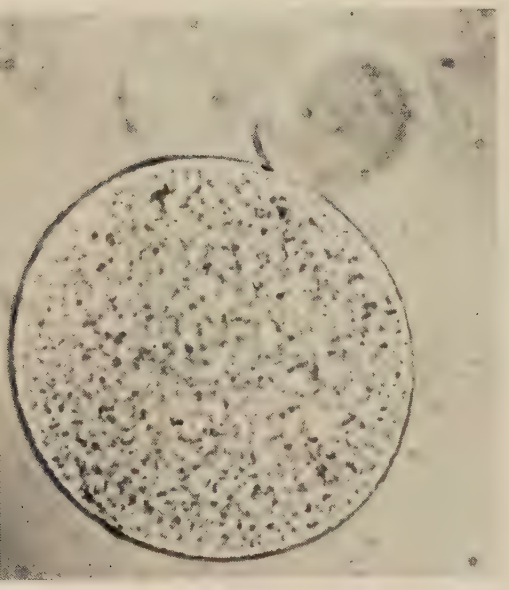


Fig. 10.

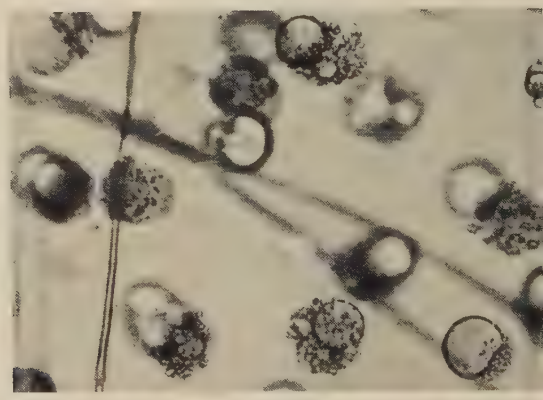


Fig. 11.

Fig. 8. Egg pretreated in normal sea water immersed in strongly diluted medium (*Echinocardium cordatum*).

Fig. 9. Eggs pretreated in normal sea water immersed in strongly diluted medium (*Echinocardium cordatum*).

Fig. 10. Egg pretreated for 8–10 hours in Ca-free sea water and thereafter immersed in strongly diluted medium (*Psammechinus miliaris*).

Fig. 11. Eggs kept for 24 hours in a mixture of equal parts normal sea water and 23 per cent sucrose solution (*Psammechinus miliaris*).

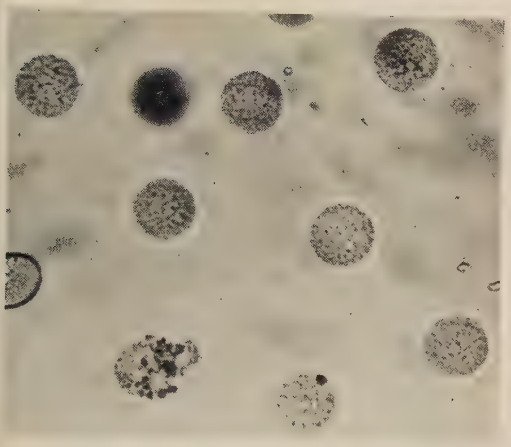


Fig. 12.

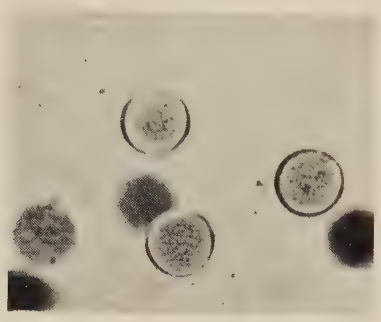


Fig. 13.

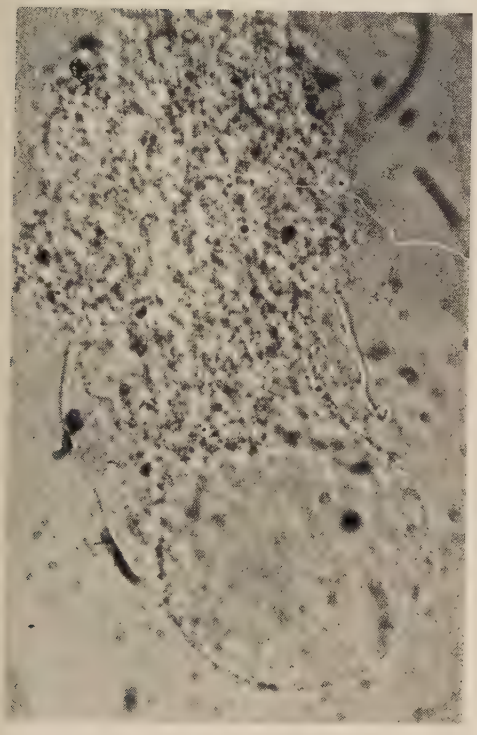


Fig. 14.

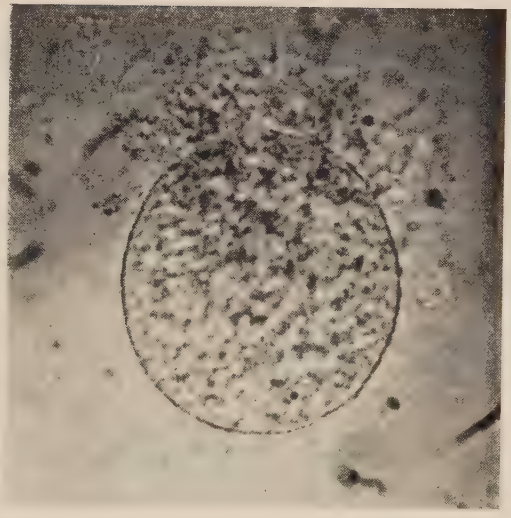


Fig. 15.

Fig. 12. Eggs kept for 24 hours in a mixture of equal parts Ca-free sea water and 23 per cent sucrose solution (*Psammechinus miliaris*).

Fig. 13. Eggs kept for 24 hours in a mixture of equal parts sea water with 0.003 M adenosine triphosphate and 23 per cent sucrose solution (*Psammechinus miliaris*).

Fig. 14. Egg pretreated with sea water immersed in strongly diluted medium. Upward in the figure the elongated cytoplasmic protrusion, below the remaining bag corresponding mainly to the vitelline membrane. At the lower part of the cytoplasmic protrusion outlines of coagulated parts of the jelly coat (*Psammechinus miliaris*).

Fig. 15. Egg pretreated with sea water with 0.003 M adenosine triphosphate immersed in a strongly diluted medium. A certain amount of cytoplasmic material has escaped from a defect in the vitelline membrane. The bulk of cytoplasm remains within the precipitated vitelline membrane (*Psammechinus miliaris*).

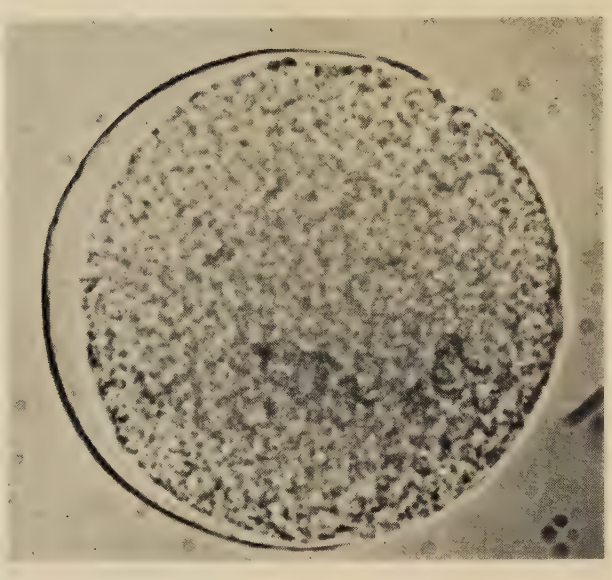


Fig. 16.

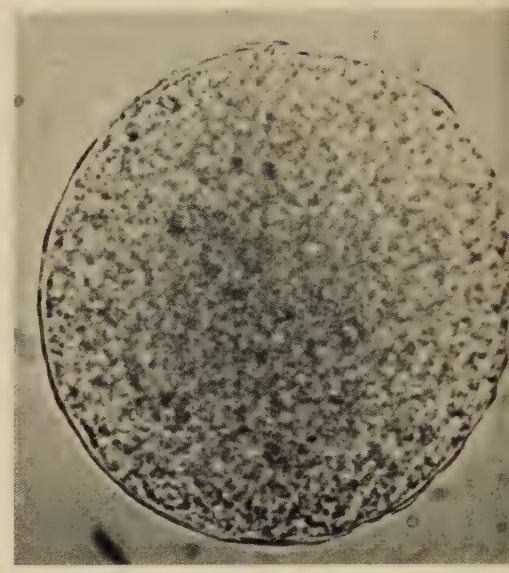


Fig. 17.

Fig. 16 and 17. Eggs pretreated with Ca-free sea water transferred to distilled water; 0.3 ml 0.01 M ATP + 0.1 ml 0.35 M $CaCl_2$ added to about 2 ml of the suspension of eggs in distilled water (*Echinocardium cordatum*).

ATP was added to normal sea water to give the concentration 0.003 M. As in experiments described in the preceding paragraph, an equal volume of isotonic sucrose solution was added. After 24 hours the eggs were still intact; see Fig. 13 which is directly comparable with Fig. 11, the control from the same experiment. The eggs in ATP resembled more those kept in Ca-free sea water + sucrose; see Fig. 12. There was, however, one conspicuous difference. The eggs in ATP had a strongly refractive surface whereas the surface of the eggs in the Ca-free medium was very weakly refractive. Under the influence of ATP a precipitation of the vitelline membrane occurred. In eggs kept in ATP in sea water, there was a tendency to precipitation of the jelly coat.

In one experiment the eggs were subjected to the action of 0.003 M ATP in Ca-free sea water. After 45 minutes they were transferred to normal sea water. When examining the eggs after about 24 hours, the jelly coat proved to be precipitated particularly in the region of the micropyle, which was rendered clearly visible under those conditions. The vitelline membrane

was precipitated and in many cases separated from the egg surface. In the control these phenomena did not occur but the eggs showed cytolysis, similar to that of the eggs represented in Fig. 11.

DISCUSSION

The present research aimed at studying the effect of ATP on the cytoplasm of the unfertilized sea urchin egg. The centrifuge experiments reported above demonstrate that addition of ATP increases the rigidity of the submicroscopic fabric of the ground cytoplasm. This must be due to a reinforcment of the bonds between the submicroscopic units, see above p. 289 concerning the interpretation of the curves in Fig. 1.

The results of the centrifuge experiments agree with results obtained when eggs pretreated with sea water + ATP or control eggs from sea water were immersed in a strongly hypotonic medium. In the former the cohesion is much higher both in the vitelline membrane and in the bulk of the cytoplasm.

When a fertilized sea urchin egg is brought into a strongly hypotonic medium it swells but retains its form. Its cytoplasm appears as a coherent framework (21). Even the cortical part retains a fine texture. In the unfertilized egg, on the other hand, the cortex breaks up upon transfer to strongly hypotonic medium, see above. The outflowing cytoplasm disintegrates into separate granules and blisters. This occurs to an extent that varies with conditions prevailing in the egg cytoplasm.

The results point to the conclusion that the cytoplasm of eggs subjected to ATP assume a structure similar to that of fertilized eggs. The effect of ATP on the vitelline membrane recalls also the changes of this membrane upon fertilization. An increased linkage between submicroscopic constituents accounts for the increased mechanical resistance. ATP may also induce an expulsion of cortical granules which then unite with the membrane. Therefore the membrane often appears granular when rendered visible in hypotonic medium. The changes brought about by ATP also interfere with normal elevation of the membrane upon fertilization of the egg (22).

In eggs centrifuged on the border between saline and sucrose solutions, a centrifugal cap of hyline material appeared. This cap was not formed when the eggs were centrifuged in a homogeneous saline medium. The contact with the sugar solution must bring about an increased density of the egg region adjacent to the sugar solution. Particularly in the presence

of ATP the change very likely resides in a parallel aggregation of fibres. The centrifugal force pulls out the fibres so that the heavier part of the egg may be strongly elongated (Fig. 3). The disappearance of the centrifugal hyaline cap after switching off the centrifuge motor is explained by the elasticity of the cytoplasmic fibres. They are probably continuous with fibres in the lighter part of the egg. When the pull exerted by the centrifugal force ceases, the fibres recover from deformation and the centrifugal hyaline cap merges with the adjacent layers of cytoplasm.

The writers were not primarily concerned with the effect of Ca on cytoplasmic rigidity, but it proved necessary to consider this problem in connection with the work on ATP. The rigidity proved to be higher in a Ca-free medium than in normal sea water. The rigidity decreased further when the Ca-content of sea water was doubled. These results coincide with those of several previous workers, see particularly (8). The texture of the cytoplasm is somewhat coarser in a Ca-free medium than in normal sea water or in sea water with increased Ca-content. In Ca-free sea water the fibrous elements of the cytoplasm form a rather rigid coagulated framework. It is of interest that not only addition of Ca but also of ATP renders the texture of the cytoplasm finer and more uniform. This may seem paradoxical, as addition of ATP increases, but that of Ca decreases the rigidity of the cytoplasm. The linkages rather than the dispersion are responsible for the rigidity of the cytoplasm. Addition of ATP increases both dispersion and rigidity. A similar change occurs subsequent to fertilization; see (23, p. 281)

A previously published diagram Runnströms (21, Fig. 11) may still serve as an illustration of the structural changes in the fabric of the ground cytoplasm occurring under different conditions including fertilization.

Eggs pretreated with Ca-free sea water and then transferred to strongly hypotonic medium represent degraded cells. They are, however, still capable of important reactions. They are parallel in this respect to the homogenates in Ca-free sea water prepared by Hultin (9, 10).

The promoted swelling of eggs transferred to hypotonic medium constitutes the most obvious effect of Ca. A great number of hyaline blisters are expelled through damaged spots of the cell surface. These blisters do not form in eggs degraded in Ca-free medium, but appear as soon as Ca is added to the medium. The blisters have the character of strongly swollen complexes of proteins and lipids. In a less swollen state, they appear as myelin figures. These may be visible in the interior of the eggs and are gradually formed even in the absence of Ca. There are thus evidences that Ca favours the swelling of certain lipoprotein complexes. These are possibly released

under the action of Ca from still larger complexes. According to Hultin (10) the lipids become more easily extractable upon addition of Ca to the homogenates prepared in Ca-free medium.

Ca also promotes a stronger coagulation of the fibrous constituents of the framework visible in degraded eggs. Ca has possibly different effects on different cell constituents, but its role in coagulation may be secondary to the release of the lipoprotein complexes. Addition of Ca has also a precipitating effect on the surface, particularly the vitelline membrane, of eggs degraded in Ca-free medium. Even a separation of the vitelline membrane from the egg surface sometimes occurs. The discussed phenomena are related to Heilbrunn's surface precipitation reaction (6) in which Ca is also involved.

According to Hultin (9) addition of Ca to egg homogenates brings about a coagulation which is revealed by an increase of viscosity. These facts agree with the results obtained with degraded eggs, but are contradictory to the effect of Ca on intact eggs.

Eggs degraded in Ca-free medium respond also to the addition of ATP with blister formation. On addition of Ca along with ATP changes are still more profound. A well marked membrane may elevate from a greater or smaller part of the egg surface. The texture of the cytoplasm simultaneously becomes finer.

The changes referred to roughly imitate what happens upon fertilization. It has been inferred (23, p. 278), that substances expressed from the egg incite the changes involved in the elevation and solidification of the membrane which occur upon fertilization. These processes require the presence of Ca and probably of ATP as well. The importance of ATP is also suggested by experiments carried out by Wicklund on *Arbacia* eggs (23, p. 251).

The changes in the membrane seem to reflect general changes which involve increased linkage between submicroscopic units in the cytoplasm.

It is difficult to understand how structure changes in the interior cytoplasm of the intact egg are brought about by low concentrations of a substance penetrating with difficulty, like ATP. According to Lindberg (15, 16) ATP is present even outside the permeability barrier of the egg. Here it easily exchanges its labile P for radioactive P added to the medium as inorganic phosphate. Admixture of ATP to the medium increases in the first place the ATP concentration outside the permeability barrier. The further penetration of ATP is probably a very slow process. ATP must therefore exert its direct action mainly on the outer part of the cortical layer. The egg is rather rich in ATP, see (23, p. 307). A possible solution of the problem would

be that a wave of ATP release in the interior of the intact egg is started when ATP accumulates in the egg surface. The same problem is met with in muscle physiology, see (1).

Problems of equal difficulty are encountered when considering the Ca-action on the interior of the intact egg. Addition of Ca to degraded eggs in which the permeability barrier is removed no doubt brings about a precipitation of the cytoplasm. Nevertheless, addition of Ca to the medium decreases the rigidity of the cytoplasm of the intact egg.

SUMMARY

The present research aimed at studying the effect of ATP on the cytoplasm of the unfertilized sea urchin egg (*Psammechinus miliaris*). For this purpose, the eggs were submitted to centrifugation. The time course of stratification was followed in a centrifuge microscope. The curves obtained demonstrate that the rate of stratification is determined rather by rigidity of structure than by viscosity. The addition of 0.003 M ATP increased the rigidity, probably by increasing the linkage between certain components of the cytoplasmic fabric.

Upon pretreatment with ATP the structure of the cytoplasm proved to be more coherent after immersion of the eggs in a strongly hypotonic medium.

The rigidity proved to be higher in a Ca-free medium than in normal sea water. The rigidity decreased further when the Ca-content of sea water was doubled.

Eggs were pretreated with Ca-free sea water and thereafter subjected to strong hypotony. When Ca was added to the hypotonic medium numerous hyaline blisters were given off from the eggs. The blisters arise by a swelling of lipoprotein complexes which in a less swollen state appear as myelin figures. Furthermore the addition of Ca causes a stronger coagulation of the egg surface and of the fibrous framework of the cytoplasm. Addition of ATP causes also blister formation in the eggs cytolyzed in a Ca-free medium. A tendency to coagulation and separation of a membrane recalling the fertilization membrane has been observed upon addition of Ca + ATP. The changes in structure caused by addition of ATP recall those occurring upon the activation of the egg.

This work has been supported by a grant from the Swedish Natural Science Research Council.

Our thanks are due to Professor Nils Holmgren and Dr Gunnar Gustafson, Zoological Station, Kristineberg, for generous help and supply of animal material, Mr. L. Ernster for preparation of the Ba-salt of ATP, and to Dr Inga Fischer for valuable discussions.

REFERENCES

1. Buchthal, F., Deutsch, A., Knappeis, G. G., and Munch-Petersen, A., *Acta Physiol. Scand.*, **16**, 326 (1949).
2. Crick, F. H. C., and Hughes, A. F. W., *Exp. Cell Res.*, **1**, 37 (1950).
3. Frey-Wyssling, A. *Submicroscopic Morphology of Protoplasm and its derivatives.* New York (1948).
4. Harvey, E. B., *Biol. Bull.*, **62**, 155 (1932).
5. Harvey, E. N., and Loomies A. L., *Science*, **72**, 42 (1930).
6. Heilbrunn, L. V., *The Colloid Chemistry of Protoplasm.* Berlin (1928).
7. —— *Amer. Journ. Physiol.*, **64**, 481 (1923).
8. Heilbrunn, L. V., and Daugherty, K., *Physiol.*, **4**, 635 (1931).
9. Hultin, T., *Arkiv Kemi, Mineral. Geol.*, **26** *A*, No 20. (1949).
10. —— *Exp. Cell Res.* **1**, 159 (1950).
11. Knaffl-Lenz., *Pflügers Arch.*, **123**, 279 (1908).
12. Kriszat, G., *Arkiv Zool.*, No. 7. **81** (1949).
13. Lindahl, P. E., *Arch. Entwicklungsmech. Organ*, **127**, 323 (1932).
14. —— *Acta Zoologica*, **17**, 179 (1936).
15. Lindberg, O., *Arkiv Kemi Mineral. Geol.*, **26** *B*, No. 13 (1948).
16. —— *Exp. Cell Res.*, **1**, 105 (1950).
17. Monné, L., *Arkiv Zool.*, **35** *A*, No. 13 (1944).
18. Öhman, L. O., *Arkiv Zool.*, **36** *A* No. 7 (1944).
19. Perry, S. V., Reed, W., Astbury, W. T., and Spark, L. C., *Biochem. et Biophys. Acta*, **2**, 674 (1948).
20. Runnström, J., *Protoplasma*, **4**, 388 (1928).
21. —— *Protoplasma*, **5**, 201 (1928).
22. —— *Arkiv Zool.*, **40** *A*, No. 1 (1947).
23. —— *Adv. Enzymol.*, **9**, 241 (1949).
24. Runnström, J., and Monné, L. *Arkiv Zool.*, **36** *A* No. 20 (1945).
25. Schechter, V., *Biol. Bull.*, **72**, 366 (1937).
26. Szent-Györgyi, A., *Studies Inst. Med. Chem. Univ. Szeged*, **1**, 67 (1943).
27. —— *Chemistry of Muscular Contraction*, New York (1947).

ANALYSIS OF THE MODIFYING EFFECT OF DISSOLVED JELLY COAT SUBSTANCE ON THE FORMATION OF THE FERTILIZATION MEMBRANE IN EGGS OF ECHINOCARDIUM CORDATUM

JOHN RUNNSTRÖM

The Wenner-Gren Institute for Experimental Biology, Stockholm

Received January, 15 1950

As shown in a previous paper (7); see also (5), the presence of heparin or dissolved jelly coat substance (abbreviated: jelly substance) modifies the formation of the fertilization membrane. The effect was particularly uniform and well defined in eggs of *Arbacia lixula*. Owing to the effect of the substances mentioned a gelatinous rim is formed around the egg upon fertilization. This rim corresponds to the fertilization membrane and also contains the cortical granules which have been extruded. The granules are not transformed — as under normal circumstances — into rods (3, 4, 5, 7) and the membrane is not separated from the egg surface. Below the membrane the hyaline layer appears. The membrane and this layer do not separate; only in the course of cleavage a tendency to partial separation may be observed. These conditions contrast strongly with those found at normal fertilization. The smooth quickly elevating membrane is separated from the hyaline layer by the perivitelline space.

It was inferred that heparin and jelly substance primarily inhibit clotting processes which are connected with the reorganization of the vitelline membrane and the conversion of the extruded cortical material.

Effects analogous to those described for Arbacia were obtained also in a number of other sea urchin species (5). It was not settled, whether heparin or jelly acts directly on the egg surface or whether the action is partly or wholly exerted on the spermatozoa. The state of the spermatozoa seems to influence the membrane formation in Arbacia. When inseminated with aged spermatozoa fresh Arbacia eggs often form a gelatinous rim instead of a normal membrane. Certain experiments indicated, however, that the spermatozoa were not involved in the inhibitory effect exerted by the jelly on the membrane formation. Despite this, more evidence was desirable.

According to the experience of this writer (6) a fertilization membrane of normal type is readily formed in eggs of the irregular sea urchin *Echinocardium cordatum* when subjected to treatment with a hypertonic solution (2 ml sea water + 0.5 ml 2.5 N NaCl).

In the other species easily available on the Swedish west-coast, *Psammechinus miliaris*, the membrane formation was incomplete upon exposure of the eggs to hypertonicity. In general the membrane remained folded and granular. The eggs of Echinocardium thus react with respect to membrane formation like the eggs of *Arbacia punctulata*. In the latter species Just found normal membrane formation and development to top swimming larvae upon hypertonic treatment (2).

No attempts were made to bring the parthenogenetic development of the eggs of Echinocardium beyond the first stages. Yet the experiments elucidate the question whether the jelly substance acts on the egg surface directly or indirectly, by influence on the spermatozoa. The membrane formation caused by hypertonic treatment in the presence of jelly proved to be inhibited in the same way as at fertilization. The action of the jelly solution is thus very likely exerted directly on the egg surface at fertilization.

The jelly solution used in the following experiments was prepared by Dr. Erik Vasseur and Mr. Walther Pudek. After repeated washings the eggs were submitted to treatment with sea water of pH 4.6. Centrifugation caused the naked eggs to collect at the bottom of the tubes. The supernatant contained the jelly solution. This solution was used in the experiments after adjustment of pH to 8.0. In previous work the jelly was generally precipitated with ammonium sulphate, dialyzed against distilled water and lyophilized. The simpler way of preparation now adopted gave less opalescent solutions. An aliquot part of the solution was dialyzed against distilled water to determine the dry matter content which amounted to 0.65 mg per ml (determination in duplicate).

THE EFFECT OF JELLY SOLUTION ON THE FERTILIZATION OF THE ECHINOCARDIUM EGG

Some experiments were carried out on the effect of jelly solution on the fertilization in Echinocardium. The results agreed with those previously found in other species. The unfertilized Echinocardium eggs are pear shaped, as described earlier (1, 4). The pointed part corresponds to the animal pole. The mass of cytoplasm is not equally distributed around the egg axis

Table I

Effect of different concentrations of jelly substance on the fertilization of *Echinocardium cordatum*. The results given in percentage of total number (n) examined.

	Spherical eggs, uniform smooth membrane	Spherical eggs, membrane low and assymetrically elevated	Angular eggs with local elevation of a low membrane	Eggs fertilized, but no membrane	n.
Control in sea water	93	2	5	0	55
0.016 per cent jelly solution..	0	0	100	0	60
0.0065 per cent jelly substance	0	12	73	7	62
0.0022 per cent jelly solution	95	3	2	0	60

Table II

Membrane formation in hypertonic solution without or with jelly substance. Unfertilized eggs were transferred to the following samples:

1. 2 ml sea water;
2. 2 » » » with 0.016 per cent jelly substance. The eggs were pretreated for 5 min. in these samples. Thereafter 0.5 ml 2.5 N NaCl added to each of them.

Time after addition of 2.5 N NaCl	1	2
1.5 minutes.	Membrane elevation begins with local bulges.	Egg surface wrinkled, cytoplasm became darker and a thin membrane was elevated.
3 minutes.	In most of the eggs smooth membrane with perivitelline space of normal width. No cytoplasmic strands extend between egg surface and membrane. In single eggs rods present under the membrane.	
4–5 minutes.	Eggs spherical. On the otherwise smooth surface a variable number of goblet like indentations, see Fig 1. The cytoplasm clear and homogeneous.	Numerous fine strands extended between the angular egg surface and the thin membrane. Cortical granules scattered in the perivitelline space, see Fig. 2. As they do not present any Brownian movement they probably adhere to the strands.

but presents rather a bilateral arrangement. Upon normal fertilization the egg becomes perfectly spherical, the original arrangement being only reflected in the membrane, the axes of which often remain unequal. Two features are characteristic of the eggs which are fertilized in jelly solution; see Table I. They do not assume the spherical form but remain more or less angular. The membrane is only locally elevated but the perivitelline

Fig. 1. Fig. 2. Fig. 3.

Fig. 1–3. Membrane forrmation in unfentilizedd eggs of *Echinocardium cordatum* upon exposure to hypertonic medium, in Fig. 1 without, in Fig. 2 and 3 with jelly solution added to the medium. The pear shape of the unfertilized egg is reflected in the form of the membrane in Fig. 1. Near the protrusion represented in the upper part of Fig. 1 two groups of rods (transformed cartical granules).

space does not even here attain its normal width. The parallel control fertilized in pure sea water presented perfectly spherical eggs with a smooth membrane which was elevated in the normal way all around the egg. Concentrations of jelly substance below 0.002–0.003 per cent gave no inhibitions.

THE EFFECT OF JELLY SOLUTION ON THE MEMBRANE FORMATION IN HYPERTONIC SOLUTION

Table II gives the results of one experiment.

Two different types could be distinguished with respect to membrane formation in eggs subjected to hypertonic sea water containing jelly solution. One of these types, referred to in Table II, is represented in Fig. 2. In another experiment carried out the visible strands extending from the egg surface were coarser and less numerous than in the eggs from the experiment recorded in Table II, 2. A number of cortical granules were attached to the membrane. Furthermore rather numerous bodies of somewhat varying diameter were visible within the vitelline space; see Fig. 3.

It has repeatedly been described how the material exuded upon activation appears in the form of globules (3, 7). The bodies represented in Fig. 3 recall these globules. They have a diameter of 4–5 μ and contain a number of cortical granules embedded in a ground substance. After 10–15 minutes exposure of the eggs to hypertonic sea water a great number of the globules became more angular. Some of them were concave-

convex. This kind of transformation of the cortical globules was observed to occur also in fertilized eggs in which the merging of the cortical material with the vitelline membrane was delayed (4, 7). The angular shape is due to a conversion of the included granules into rods. By comparing the two types in Figs. 2 and 3 respectively it seems probable that the strands and the ground substance of the globules represent two different appearances of the exuded material, in which the cortical granules are embedded.

SUMMARY

Addition of a solution of jelly coat substance inhibits the normal development of the fertilization membrane upon insemination of *Echinocardium cordatum* eggs. The presence of jelly also prevents the egg from assuming the smooth spherical shape, characteristic of normal fertilized egg.

A fertilization membrane of normal character appears in Echinocardium eggs upon exposure to a hypertonic medium (2 ml sea water + 0.5 ml 2.5 N NaCl solution). The presence of jelly prevents the completion of the artificially induced membrane formation. The cortical material does not merge in the normal way with the vitelline membrane. As a consequence the fertilization membrane remains thinner than in eggs treated with jelly free hypertonic solution. The outline of the membrane is also more irregular. The egg is angular and the cytoplasm opaque (Figs. 2 and 3). This contrasts with the smooth spherical form and clear cytoplasm of the eggs subjected to hypertonic medium without jelly.

The evidence presented favours the conclusion that the dissolved jelly substance inhibits membrane formation at fertilization by direct action on the egg surface rather than by influence on the spermatozoa.

This work was supported by the Swedish Natural Science Research Council. Thanks are due to the authorities of the Kristineberg Zoological Station, Fiskebäckskil, for generous help and supply of material.

REFERENCES

1. Gustafson, T., *Arkiv Zool.*, **37** *B*, No. 1 (1945).
2. Just, E. J., *Biol. Bull.*, **43** (1922).
3. Runnström, J., *Arkiv Zool.*, **40** *A*, No. 1 (1947).
4. —— *Arkiv Zool.*, **40** *A*, No. 19 (1948).
5. —— *Advances in Enzymol.*, **9**, 241 (1949).
6. Runnström, J., Monné, L., and Wicklund, E., *J. Colloid. Sc.*, **1**, 421 (1946).
7. Runnström, J. and Wicklund, E., *Arkiv Zool., N. S.*, **1**, No. 13 (1950).

SHEATH AND AXON STRUCTURES IN THE INTERNODE PORTION OF VERTEBRATE MYELINATED NERVE FIBRES

AN ELECTRON MICROSCOPE STUDY OF RAT AND FROG SCIATIC NERVES

H. FERNÁNDEZ-MORÁN

Department of Cell Research, Karolinska Institutet, Stockholm

Received January 1, 1950

A. INTRODUCTION

Fundamental problems of nerve fibre structure have proved difficult to solve with the classical methods of light microscopy, partly because nerve colloidal organization is extremely sensitive to chemical and physical agents, but also due to the limited resolving power of the microscope. The need for direct visualization of details lying beyond the light microscope's resolution is hardly more manifest than in this case, where no one has yet clearly seen the physical relation of the myelin sheath to the axon or determined the fine structure of these two main components of the nerve fibre by direct observation. Considerable information on the ultrastructure of nerve has already been obtained from polarized light and X-ray diffraction studies, but only direct access to the submicroscopic domain can confirm the interpretation of these data and elucidate the details of the fibre's organization.

Successful application of the electron microscope to this problem depended on the development of suitable thin sectioning techniques, since the commonly used fragmentation procedures yield isolated elements which are difficult to trace back to their original position within the fibre. The introduction of new methods for cutting ultrathin sections by Pease and Baker (28) and by Newman, Borysko and Swerdlow (26) now makes this approach possible. By using a modification of the thermal-expansion technique of Newman, Borysko and Swerdlow (26) extremely thin (0.3–0.1 μ) *frozen sections* of unembedded, medullated nerve can be cut (Fernández-Morán, 17). This method has been improved by applying a motor drive to the microtome and a special collecting reservoir attached to the knife, so that *ultrathin serial*

frozen sections, practically free of distortions, are obtained routinely. In this way sections comparable to the usual embedded ones, but which have not been subjected to preliminary treatment with alcohol, ether and the other embedding manipulations, are made available for electron microscopic examination. This advantage of frozen sections also makes possible the use of metallic shadow casting to enhance contrast without having to dissolve the embedding matrix, thus eliminating the repetition of an extraction process which may affect the fine structure of nerve. Extensive work on frozen sections was therefore carried out, supplemented by examination of nerves embedded in collodion-paraffin, in gelatine, and combined with replica-adhesion sections of dissected single fibres and with a variety of fragmentation procedures.

These studies have revealed interesting structural details of the myelin sheath and the axon, determined the location and relationship of the different components with greater clarity, and yielded a general picture of the nerve fibre organization in the internode segments which will be described in this report.

B. PREVIOUS WORK WITH THE LIGHT MICROSCOPE

Only the more important observations relevant to this investigation will be summarized here. The living vertebrate nerve fibre appears essentially as a pearly glistening soft cylinder of myelin, usually 2–16 μ in diameter, enveloped by membranous sheaths, with a rod-like gelated core or axon, and interrupted at periodic intervals of 1–3 mm by constrictions or Ranvier nodes. These periodic nodes and frequent incisures in the internode portion (the Schmidt-Lantermann incisures of fixed preparations) are the only constantly discernible structures in the normal, fresh fibre, since both sheath and axon are devoid of microscopic details in visible light (de Rényi, 9) under dark field examination (Ettisch and Jochims, 14; Auerbach, 3), in polarized light (Schmidt, 33) and also in ultraviolet light according to Massazza (22). v. Muralt (24) however describes a distinct network visible in isolated living frog nerve fibres examined with short wavelength ultraviolet light. Fixed and stained nerves reveal a different, more complicated picture. The axon differentiates into fibrous structures: the neurofibrils, and the myelin sheath is shown bounded by external fibrous layers (Henle's sheath and the sheath of the Schwann cell or Neurilemma) and by an internal continuation which delimits the myelin from the axis cylinder, called Axolemma. The

myelin sheath exhibits the Schmidt-Lantermann incisures which, according to some histologists, contain a special cementing substance (Cajal, 29) binding the resulting cylindroconic segments together.

Suitable staining will reveal structures known as Golgi funnels with the spiral apparatus of Rezzonico in the incisures. Since we are concerned with the internode portion, a discussion of the complicated structures described in the nodes will be omitted. Depending upon the choice of histochemical techniques the substance of the myelin sheath presents different aspects. Of particular interest is the separation of the myelin sheath into concentric lamellae observed by Nageotte (25) and by other histologists after fixation with potassium bichromate and acetic acid. Nageotte (25) describes the lamellar composition of the sheath in great detail and even assumes that each of the thicker lamellae consists of a certain number of unit lamellae, but he also refers to lipoidal rodlets extending from the inner to the outer surface of the cylindroconic segments. Lipoid extraction of the sheath usually produces a network of protein (neurokeratin). Microdissection experiments on living nerve fibres of the frog carried out by de Rényi (9, 10) are particularly valuable in connection with electron microscopical studies, because the results of micromanipulation point to certain structural relations which can be checked with this technique. By comparing freshly isolated nerve fibres with those of the frog nictitating membrane a relation of 1: 6 or 1: 7 between the diameters of the myelin sheath and of the axis cylinder, corresponding to a ratio of a 1: 1 in cross-sectional areas, was set up as characteristic for normal nerve fibres. The tissue surrounding the nerve fibre (Henle's sheath) is extremely adherent to it, but can be separated by microinjection of saline solution which removes the sticky interfibrillar substance. The fibrils appear to be elastic. A naked axis cylinder stripped of its myelin sheath keeps its shape, has a soft jelly-like consistency and, according to some experiments, is elastic to a certain extent. The myelin sheath slips on the axis cylinder when pulled, leaving myelin droplets attached to it, which indicates the existence of organic connections between the two components. Injection of saline into the fibre with a micropipette separates axis cylinder, myelin and neurilemma, the latter two with particular ease. De Rényi (10) believes that the axolemma is absent from living fibres.

Polarized light studies. As Schmitt (35) points out, Ehrenberg's (12) discovery of nerve birefringence a century ago marks the beginning of our knowledge of its ultrastructure. Subsequent investigations by Valentin (45), Klebs, v. Ebner (cit. 19), Ambronn (1) and specially by Göthlin (19), later the fundamental work of Schmidt (31, 33, 34) Schmitt (35), Schmitt and Bear (38), Chinn and Schmitt (8), Bear, Schmitt and Young (5), have considerably extended the information which can be gained by polarization optical analysis.

The embedded myelin is responsible for the birefringence of the sheath, which is positive referred to the radial direction. The anisodiametric optically positive lipoid molecules comprising the myelin are therefore considered

to be oriented radially and perpendicular to the fibre axis. Dissolving the lipoids by treatment with alcohol reverses the sign of birefringence, this effect being ascribed to the negative uniaxial birefringence contributed by the protein components of the sheath (Mezzino, 23; Schmidt, 33; Chinn and Schmitt, 8). Immersion experiments show that the protein birefringence is mainly textural birefringence, of negative sign and maintaining the same radial orientation as before extraction. According to O. Wiener's theory this would correspond to particles with their long dimensions parallel to the surface of the sheath, and it is assumed that the proteins are organized as platelets or lamellae (Schmidt, 33; Chinn and Schmitt, 8). The marked tendency of the myelin sheath to cleave into concentric lamellae (Nageotte, 25) seems to support this view. Upon fixation, the neurokeratin network presumably derives from this protein, but on the whole we know too little about this protein component, so that according to Schmitt (36) "it is impossible to say anything about its configuration in the very thin layers in the sheath".

Based on polarized light studies, Schmidt (34) assumes that the myelin sheath is built up of concentrically arranged neurokeratin lamellae running tangentially, alternating with bimolecular lipoid layers.

The fresh axon shows weak positive uniaxial birefringence indicating the presence of elongate particles. These must have considerable regularity of internal structure although they occupy a small part of the axon volume, according to an analysis of the measurable positive form birefringence of the axon of squid giant fibres (Bear, Schmitt and Young, 5).

C. X-RAY DIFFRACTION STUDIES

This method of ultrastructural analysis offers the advantage of operating on whole nerves, and the amount of radiation necessary seems to have negligible effects upon the irritability and action potential of nerve (41). The X-ray diffraction pattern of a fresh vertebrate nerve corresponds very nearly to "that which would be obtained from an internodal segment of a single nerve" (Schmitt and Bear, 39). The first studies on nerve by this method were made by Herzog and Janke, Handovsky and Thiessen (20) and by Boehm (7). But the most extensive and fruitful investigations were performed by Schmitt, Bear and Clark (41), Schmitt and Palmer (40), Bear, Palmer and Schmitt (6), Palmer and Schmitt (27).

The patterns obtained from a variety of fresh vertebrate myelinated nerves are essentially similar and can be attributed entirely to the myelin sheath,

since they are nearly completely reproduced by benzene extracts of nerves rubbed with water (41). Long spacing equatorial diffractions of amphibian (frog spinal roots and sciatic) nerves were obtained, which appeared to be second, third, fourth and fifth orders of a unit spacing of 171 Å. Mammalian medullated nerves showed an identity period of 184 Å. It is pointed out that this fundamental unit spacing is the smallest value recorded, and the possibility of a higher organization admitted, which might be revealed by improved methods (41). In the patterns of dried nerve a spacing of 144 Å (amphibian) and of 158 Å (mammalian nerve) was recorded directly. Regarded as orders of a single identity period, the long-spacings of fresh nerve show a marked alternation of intensities of the even and odd orders, which has not been observed in the patterns of dilute emulsions of mixed lipids containing no protein (27). Neither can the long-spacing of dried nerve be reproduced by such dried lipid emulsions, which only show the spacings characteristic of the double layers of the separate phases of dry lipides.

Schmitt, Bear and Palmer (42) therefore conclude: "that only the presence of protein in the repeated fundamental structure of the myelin sheath can account for the 140–160 Å period of dried nerves." Meridionally accentuated short spacing reflections at 4.7 Å and (weak) at 9.4 Å correspond to the random distribution of the lipoid molecules in the tangential direction, indicating the average interchain separation of the hydrocarbon chains of the lipoid molecules (41). The fundamental long-spacing diffraction can be interpreted in terms of radial sheath structure by assuming that it corresponds to the thickness of the concentric lipid-protein layers derived from polarized light studies. The value of 159 Å for dried mammalian nerve would result from approximately 134 Å of double lipid layers and 25 Å protein (42). Deducing the general features of the sheath's ultrastructure from the available polarized light and X-ray diffraction data on that part of the sheath which exhibits a high degree of organization, Schmitt (36) arrives at the following conception: "Essentially the sheath consists of lipid-protein layers about 180 Å thick wrapped concentrically about the axon." The 25–30 Å thin protein sheets are interspersed between double lipid layers, while the smectic mesomorphic double layers of mixed lipids have radially oriented paraffin chains.

D. PREVIOUS ELECTRON MICROSCOPE WORK

This brief survey clearly indicates the unique degree of molecular organization which we can expect to find in the nerve fibre with the help of electron microscopy. Actually, investigation of nerve structure with this instrument

has not progressed beyond the study of certain isolated elements in fragmented preparations. Richards, Steinbach and Anderson (30) observed contorted fibrillar structures composed of elongate particles aggregated linearly in extruded squid giant fibre axoplasm. De Dobertis and Schmitt (11) described fibres with dense edges, low density in the central axis and a periodic cross banding in fragmented formalin fixed nerves of different types. These fibres, tentatively called "neurotubules", were located in the axon based on indirect evidence. Recently, Schmitt (37) described a fibrous component of formalin-fixed axons of squid giant fibres consisting of a reticulum of nodose fibrils 200–400 Å in width. Extruded squid axoplasm yields fibrous structures which after blending and formalinization closely resemble the fibrous component isolated from formalin fixed fibres.

Fernández-Morán (16) applied a modified replica-adhesion method to isolated, air-dried medullated frog sciatic fibres and observed a trabecular fibrous network surrounding the axis cylinder and its longitudinally arranged fibres which he regarded as similar to the neurotubules. Sjöstrand (44), de Robertis and Schmitt (36) observed thin layers in preparations from fragmented myelinated nerves. These fragmented layers may have been derived from the myelin sheath, but Schmitt (36) points out that the fragmented layers frequently show an unexpected characteristic angular cleavage. Fernández-Morán (17) examined thin frozen sections of osmic acid fixed rat medullated nerve fibres and described a central lamellar part of the sheath which is enclosed by internal and external fibrous layers. The main central part of the sheath breaks down into piles of thin, flexible lamellae 100–150 Å thick.

E. MATERIALS

The sciatic nerves of 14 albino rats and 10 green frogs were examined, in addition to control samples of motor roots, medulla sections and optic nerves taken from each group. The animals were killed by swift decapitation or the nerves dissected out and removed under ether narcosis. Special care was taken to preserve the normal position and length of the nerves as far as possible. Usually one sciatic nerve was ligated and affixed to special glass or paraffin coated wood rods before severing off the ends with a sharp razor, while the other sciatic was stretched out gently on a glass rod without ligating and immersed directly in the fixation fluid. In a number of cases fixation in situ was tried by applying cotton or glass wool pads soaked with the fixation agent before extracting the nerve.

The following fixatives were used:

a. Mainly 1 per cent and 2 per cent osmium tetroxyde solution prepared according to *Romeis* with 0.1 per cent chromic acid. Fixation was carried out in the cold

(4° C) or at room temperature, preferably in the former, and during periods ranging between 24 to 96 hours.

b. Formalin, in 4 per cent and 10 per cent neutral solutions.

c. Fleming's chromic acid-osmium-acetic acid solution.

d. Ammonium molybdate in a 5 per cent solution according to Bethe-Dogiel, used for control purposes. Fresh, unfixed nerves sectioned with the freezing microtome (after deep freezing of knife) and picked up on saline or on glass slides, were used for replication after fixation with formalin or osmium vapors.

Individual experiments like alcohol extraction of whole nerve and incubation in trypsin will be described separately.

F. METHODS

The following preparation techniques were employed:

1. *Thin frozen sections of unembedded nerve*

In this modification of Newman, Borysko and Swerdlow's method, cooling with carbon dioxide is used to operate the thermal expansion advance mechanism and at the same time to freeze the wet specimen, so that thin sections can be cut. A standard Spencer rotary microtome model No. 820 fitted with an electric motor drive to deliver 50–100 microtome wheel revolutions per minute was used. Both the thermal expansion device described by Newman, Borysko and Swerdlow (26) or a conventional freezing microtome stage, firmly attached to the specimen holder, can be employed. Schick razor blades (38 ×8 mm) give satisfactory results and can be easily replaced and discarded after use. (Special thick Eskilstuna razor blades were also used.) Both types of blades are fastened in a specially constructed knife holder (k) (Fig. 1). A nozzle for deep freezing of the knife holder is also provided. In the first experiments individual sections were picked off from the knife edge with wooden needles. Later, however, a small cup (15 mm in diameter) was attached to the holder and filled with distilled water, saline or glycerine, thus enabling the sections to float off from the knife edge and spread out the moment they touched the liquid surface.

Operation: A nerve segment not exceeding 1–2 mm in length and 1 mm diameter is cut off from the well rinsed, fixed whole nerve and suitably oriented on a thin wooden block (10 ×3 mm) glued to the stage of the freezing microtome with a 8 % gelatine solution. Direct contact of the nerve with the metal stage is unfavourable, because it often leads to disturbing ice formation

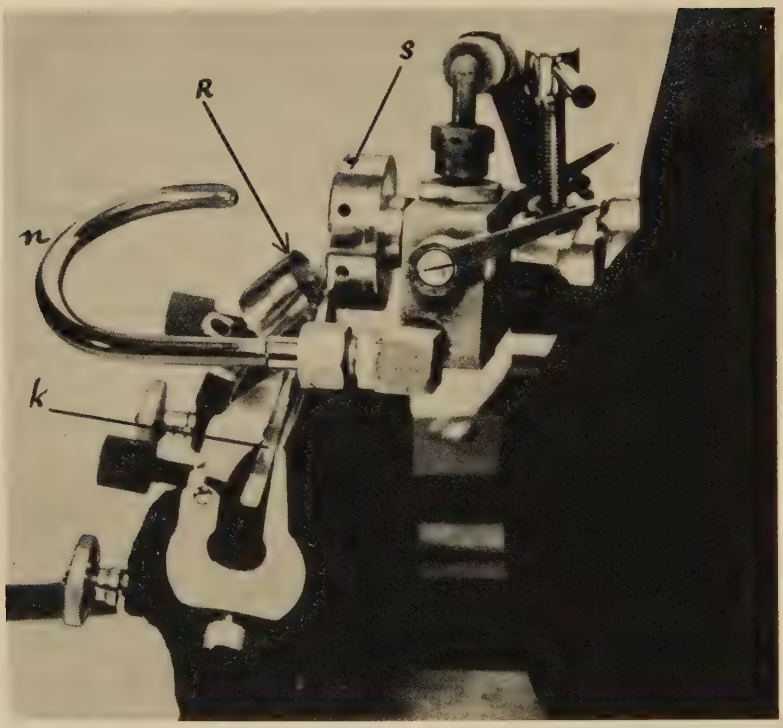


Fig. 1. *Thermal expansion apparatus* used for cutting ultrathin frozen sections of unembedded nerve, attached to a Spencer rotary microtome equipped with a motor drive. The reservoir cup (R), filled with water and attached to special knife holder (K) is shown; also a conventional freezing microtome stage (S) and nozzle (N) for freezing knife.

around the specimen. Firm adherence of the specimen is secured simply by freezing the moist nerve on to the wood base. The well frozen nerve is advanced until it comes in contact with the knife edge and a series of 1 μ sections cut by turning on the motor drive. Provided adequate knife tilt and proper adjustment of the water level are maintained (meniscus preferably 0.2 mm below knife edge at the start, with plane or slightly concave water surface), the sections will float off and adhere edgewise to form a thin ribbon which advances steadily towards the operator with each cutting stroke. It is now merely necessary to disengage the microtome's mechanism by setting it at zero, and to let the continuous thermal expansion of the stage advance the specimen by fractions of a micron, in order to obtain a tenuous ribbon of ultrathin serial sections. The sections are 0.3–0.1 μ thick, sometimes even less, depending upon the degree of specimen freezing, the

sharpness and tilt of the blade, the smooth operation of the feeding and collecting mechanism, and upon variable mechanical factors operating within the microtome itself and the motor drive. Knife tilt is different for each specimen and critical, since it can mean the difference between a clean slice or various degrees of specimen fragmentation. Proper consistency of the frozen specimen (which must always be free of ice) and correct adjustment of the meniscus in the water reservoir are equally decisive. These factors are numerous and of course important, but in practice the method has been found to work fairly constantly and smoothly, giving sections with a minimum of distortion which are comparable to those obtained from embedded material, since surface tension and the gentle push of succeeding sections are the main forces involved, direct interference of the operator being eliminated.

Once a ribbon of suitable length (10–20 serial sections) has been cut, the drive is turned off, and the sections picked up by the simple expedient of lowering a collodion covered electron microscope grid parallel to the water surface until the film touches the sections which will immediately adhere to it, flattened and undistorted. The wet sections adhere firmly to the collodion film and the final drying therefore occurs in an extremely thin tissue slice which is extensively supported by a plane membrane. The sections are now ready for staining (with 0.1 per cent phospho-tungstic acid), metal shadow-casting (with platinum-palladium at an angle of 5–20°) or direct examination.

2. *Frozen sections of nerves embedded in gelatine*

Osmium and formalin fixed nerves were embedded in 10 per cent and 20 per cent gelatine solutions according to the method of Heringa and ten Berge. Thin frozen sections of this embedded material are easily cut with the procedure described above, but they offer no distinct advantages as regards better preservation of the specimen. Instead, it was found difficult to remove the last traces of gelatine which obscure fine detail in the sections. Gelatine embedding is mainly valuable as a control method.

3. *Thin sectioning of collodion-paraffin embedded nerve*

Fixed nerves were embedded in collodion after dehydration in an ethyl alcohol series and infiltrated with 58° C paraffin, following the instructions of Pease and Baker (28). Dehydration of nerves with glycerine instead of alcohol according to Wolf's (46) glycerine-celloidin method gave superior results. Thin sections of this material were cut with the thermal-expansion device, using the collecting reservoir filled with an alcohol/glycerine mixture or with warm water. The sections are transferred to screens covered with thin aluminum or beryllium films, where the final dissolving of the embedding medium is carried out.

4. *Replica-adhesion sections of dissected nerve fibres*

(Fernández-Morán, 16, 18). Single fibres isolated by dissection of fresh nerve on a glass slide under the binocular microscope, were fixed with osmium or formalin vapors. After drying they are covered with a 0.2 per cent collodion solution in amylacetate and half an hour later with a 1 per cent solution. The resulting thick collodion film is then stripped off, carrying an extremely thin section of the fibre's surface layers, while the bulk of the fibre is left behind, allowing the procedure to be repeated. The thin section lodged in the collodion film is shadowed with platinum-palladium, followed by vertical deposition of a thin aluminum film, and finally examined after dissolving the collodion. This procedure is of special value in determining the surface structure of nerve fibres.

5. *Fragmentation of nerve fibres*

Freshly isolated fibres fixed with osmic acid or formalin were fragmented in a microblender and the centrifuged suspensions examined. In most cases, however, fragmentation was carried out by light tapping on osmic acid fixed nerve preparations enclosed between glass slide and cover slip. This simple method permits direct observation of the fragmentation process, and is specially efficient when applied to thin frozen sections.

An RCA EMU electron microscope using a saturated emission type electron gun and equipped with an RCA telescopic image viewing device and with two additional objective lens control rheostats (total variation $\frac{1}{10}$ and $\frac{1}{100}$ of focus control III), was used in this study. Compensation of the objective was also carried out according to the procedure described by Hillier and Ramberg (21).[1]

G. RESULTS

I. *Structure of the myelin sheath of rat nerve*

The general features of the sheath structure are studied best in transverse sections. The myelin sheath appears as a dense ring in 1–2 μ thick frozen sections of osmium fixed nerve, but the higher resolving power of the electron microscope readily discloses two fibrous envelopes or belts which slip off from the ring. (Plate I a.) The external girdle consists of a granular membrane with interlaced collagen fibrils which form a continuous palisade around the outer circumference of the sheath and probably corresponds to

[1] The author wishes to express his sincere thanks to Dr. Hillier and Dr. Ellis for their valuable advice and for practical demonstration of the compensation procedure.

the neurilemma with adhering connective tissue fibres. The internal membrane is formed by a dense network of much finer fibrils with longitudinally arranged broad reinforcements. This membrane will be described in detail later.

a. THE MYELIN LAMELLAE

In thinner or slightly fragmented sections the main body of the sheath dissociates into concentrically arranged rings (Plates I b and IV a) from which thin ribbons are seen to peel off. The shape of the lamellae is determined by the plane of section, since in transverse sections round bands of widths corresponding to the thickness of the section are found, while long thin ribbons or corrugated laminated slabs of thin plates are typical of longitudinal sections. Larger lamellar pieces can be isolated by gentle fragmentation of osmic acid or formalin fixed whole nerves or of thicker frozen sections. They are not preserved as well by formalin fixation which often gives them a peculiar "molten" or liquid appearance with soft, rounded edges and numerous droplets. The myelin droplets released from fragmented fresh nerves show similar structures after formalin or osmium fixation. In preparations fixed with osmic acid they resemble transparent, flexible films, with numerous folds, angular edges and appearing no thicker than the collodion specimen film. Dense, granular osmium precipitates frequently cover the lamellae, but they show no distinct structure of their own. After light metal shadow casting they exhibit a characteristic structure, quite different from the background film structure, and consisting of 30–90 Å high closely packed granules (Plate III a). In some preparates only droplets of variable size (100–200 Å diameter) are seen on the otherwise smooth lamellar surfaces (Plate II a). Usually the lamellae are found piled closely upon each other (Plate II b) in slabs reminiscent of mica plates, with angular edges of uniform thickness arranged staircase-like. 6, 8 and even 10 lamellae can be distinguished in a single pile. The thinnest, or unit lamellae, disintegrate into granules in certain areas, resulting in finely perforated, porous lamellar patches found even in well fixed preparations. The accuracy of measurements carried out on shadowed specimens (Table I) is limited by the resolving power of 20–30 Å of our microscope, by the thickness of the deposited metal layer and by the difficulty in determining the exact orientation of the shadowed edge. Within these limits the unit lamellae have an average thickness of 50 Å, without the granular surface structure which is unequally distributed and contributes 30–100 Å to give a total thickness of the lamellae varying between 80 and 150 Å. *The lower value of 80 Å predominates.*

TABLE I

Thickness of the myelin lamellae and of their surface structures.
(Each figure represents the mean value of 10 measurements made on single lamellae.)

Rat sciatic		Frog sciatic	
Lamellae Thickness in Å	Surface structures Height in Å	Lamellae Thickness in Å	Surface structures Height in Å
50	30	50	50
40	40	40	90
50	60	30	60
50	100	60	40
40	60	40	40
60	70	50	30
70	80	30	70
60	70	30	90
30	60	40	40
50	50	40	50
Mean: 50 Å	Mean: 62 Å	Mean: 41 Å	Mean: 56 Å

b. THE CONCENTRIC LAMINATED STRUCTURE OF THE SHEATH

Although there are definite indications that the bulk of the sheath separates into concentrically arranged rings which consist at least in part of thin lamellae, only thin sections can show if there are other structures besides the lamellae in the sheath, and how the latter are arranged. Transverse sections thinner than 0.5 μ show the characteristic onion peel-like concentric lamellated structure (Plate IV a) and appear riddled with submicroscopic oval crevices (0.1–0.4 μ). These crevices result from regularly occurring gaps between the individual layers. They are seen equally well in transverse and longitudinal frozen sections and their distribution is variable. Sometimes half of the myelin sheath is occupied by them while the other half looks solid, but generally they cover the entire tube wall. Since they are not seen with equal frequency in collodion-paraffin embedded nerves, they may be desiccation artefacts. Their constant occurrence suggests, however, that they can be naturally existing submicroscopic crevices filled with liquid, or that the attachment of the concentrically arranged lamellar bundles is less firm in these spots.

Still thinner sections (0.2–0.1 μ) exhibit a distinct laminated structure within the single concentric layers. The laminated structure is seen throughout the well

preserved parts of the sheath wall in shadowed preparations (Plate V a), while unshadowed sections show the lamination clearly only in suitably oriented places, where slight separation of the lamellar sheets has occurred. In general, the lamination is best seen in frozen sections because of the characteristic loosening and separation along potential cleavage planes produced by desiccation. The laminated areas show a regular arrangement of concentrically stacked up layers which sometimes appear as clear cut lines (Plate V b) but more often seem to consist of compact granular rows (Plate V a and VI a). This granular appearance obliterates part of the lamination, making it difficult to measure the thickness of the individual layers. *Determination of this average thickness in a large number of preparates gives values ranging between 70 Å and 140 Å, corresponding to the thickness of 1–2 unit lamellae.* The layers follow the contours of the crevices closely and exhibit uniform thickness and spacing (Plate VII a).

The same type of lamination, longitudinally oriented, can be observed extending uninterrupted throughout large segments of the myelin sheath in longitudinal sections. Folding and bending of the thin longitudinal sections and occasional appearance of thicker areas or invaginations in the myelin sheath wall made it impossible to trace the course of a single layer in sections longer than 2–3 microns. Discontinuities or incisures in the sheath could therefore not be recognized and more material must be studied in order to solve this problem. Tangential or longitudinal sections which pass through the sheath in a plane parallel to the concentric layers demonstrate a typical corrugated and ruffled structure, resulting from the rumpled edges of the myelin lamellae, lifted out of their place by the knife. Here again the oblong sheath crevices, resembling troughs, can be seen. Since several hundred transverse and longitudinal sections examined show essentially the same type of concentric lamination and configuration, it is assumed that this represents the general structural pattern of the sheath in the internode portion.

c. The concentric grating structure in thin sections of the sheath

In sharp contrast to the comparatively indistinct concentric lamination of the sheath is a structure of remarkable regularity and clearness which was constantly found associated with the laminated layers and can best be described as *a concentric grating system.* This grating was seen only in osmic acid fixed transverse and longitudinal frozen sections, where it appears as *a regular system of lines arranged concentrically in the sheath, parallel to the laminated layers or superimposed upon them.* The lines occur predominantly

TABLE II

Spacings of the concentric line system superimposed on the laminated structure of the myelin sheath.

(Each figure represents the mean value of 10 measurements made on the line systems of different preparates.)

Rat sciatic	Frog sciatic
70	50
70	60
60	55
70	65
80	50
80	50
70	60
60	55
70	65
80	70
Mean: 71 Å	Mean: 58 Å

in lightly (platinum-palladium) shadowed, extremely thin (0.1–0.07 μ) sections in variable quantity, numbering from 4 to 50 or more in continuous succession. The distinguishing feature is the extremely uniform, precise configuration of the lines and the perfect regularity of their spacings. (Plates VI b, VII b, c, VIII a, b.) The spacings measured in transverse and longitudinal sections of rat and frog sciatic are given in Table II. This striking regularity leads one immediately to suspect that it may be some type of electron optical diffraction phenomenon. Closer examination reveals that they are different from any of the diffraction fringes or similar effects recorded in the literature, both as regards their number, regular spacing and uniform width and intensity. Moreover, they follow even the most complicated contours of the sheath laminae very closely, never extending beyond the clear cut borders of these structures. They are seen only in certain suitably oriented parts of the specimen, as can be shown by stereoscopic pictures. The grating phenomenon is very sensitive to focussing, appearing only in those plates which are in correct focus (judging from the images of adjacent holes in the collodion film) and vanishing completely if the objective setting is slightly changed. The finest details of surrounding areas of the film are clearly visible, and specimen contamination as a possible source seems therefore improbable. The large number of consecutive lines and the characteristic

way in which they blend with the coarser laminar structure, continuing it and arranged always concentrically around the fibre axis, provide further evidence against their being a spurious diffraction phenomenon. On the other hand, the frequent occurrence of this grating system in certain parts of the specimen which are slightly thicker than adjacent thin areas devoid of the phenomenon, leaves certain doubts as to their nature. Besides, it is not clear how such a regular system of lines can be produced by direct transmission through a relatively compact structure supposed to consist of alternating layers of lipids and protein, both of which stain more or less intensely with osmic acid. Even assuming that the layers have been cut thin enough to appear partially transparent and that the slight separation of lamellae resulting from vacuum desiccation facilitates beam transmission, slight tilting or warping of the individual lamellae suffices to affect the regularity of the grating system or to extinguish it altogether. *Nevertheless, the average value of 70–80 Å determined for the line spacing would correspond approximately to the thickness of one unit lamella.*

This phenomenon has been the subject of such extensive discussion because it cannot be dismissed lightly as a spurious effect, since it is remarkably constant in appearance and parallels the concentric laminated structure of the sheath too closely. At present it is not possible to explain the grating effect satisfactorily or to evaluate it as further proof of the myelin sheath's foliate structure. This matter is being further investigated, but tentatively the view is held that the described grating phenomenon corresponds to an underlying regularity of the sheath's ultrastructure which can be studied when this effect is better understood.

This grating system is clearly different from the diffraction effects occasionally observed in piles of myelin lamellae lying flat on the specimen film. The series of lines and the peculiar wavy dark bands observed in these piles bear a certain resemblance to the patterns described as electron optical effects in split mica. Electron diffraction studies on the fine structure of the myelin sheath, specially with the convergent beam method of Kossel and Möllenstedt, might yield valuable complementary information.

The thinnest sections in which the laminated structures can be made out are rarely intact and slightly fragmented due to cutting effects. Therefore, it was not possible to determine the radius of the corresponding nerves with sufficient accuracy. Differences in fine structure which might occur between large and small nerve fibres could not be investigated in this preliminary survey.

d. COMPLEMENTARY STUDIES ON THE STRUCTURE OF THE SHEATH

1. *Results of lipoid extraction.* Alcohol extraction of whole nerve was tried in an attempt to isolate the protein component of the sheath by removing most of the lipoids. Ligated and mounted rat and frog sciatic nerves were run up through a graded series of alcohol and transferred to absolute alcohol for 24 hours. This was followed by immersion in frequently renewed ether solution (12 hrs.), and the extracted nerves were finally fixed in 1 % osmic acid (24 hrs.). Examination of thin (0.3–0.2 μ) frozen sections of the extracted nerves with the electron microscope reveals a *large number of thin, transparent leaflets* loosely separated from the more compact concentric layers. These leaflets, appearing singly or in stacks of 3–6, are similar to the lamellae seen in normal osmic acid fixed nerve.

In shadowed preparations they are smooth, look thinner and exhibit little of the granular surface structure typical of unextracted lamellae. Measurements of their thickness yield values ranging between 30 and 40 Å. Presumably, removal of the binding lipoid substance between the lamellae has resulted in the extensive release of these films which are either protein membranes or consist of a stable lipid-protein complex resistant to alcohol ether. Since no other satisfactory way of dissolving the lipoid component of the sheath lamellae without disfiguring them is available, this experiment indicates that extraction of lipoids leaves behind certain lamellar structures in the sheath, which are similar to the normal myelin lamellae devoid of granular surface structure, and probably represent protein films.

2. *Digestion experiments with trypsin.* The opposite type of experiment: digestion of part of the protein components of the sheath by prolonged treatment with trypsin, was also carried out. Whole fresh rat and frog sciatics were incubated in a 0.3 % solution of crude trypsin in a 0.4 % Na_2CO_3 solution or in phosphate buffer (pH 8) at 37° C for 7 days. The nerve was then quickly rinsed in saline and transferred to a 1 % osmic acid solution. A piece was cut off after 24 hours, single fibres isolated and fragmented gently between slide and cover slip. The other half was sectioned with the freezing microtome. In both cases characteristic myelin lamellae (Plate III b) are obtained with a peculiar crumbling appearance or fenestration. This cribriform structure is due to disintegration of the ordinarily compact lamellae into composite granules consisting of 50–100 Å high, rod shaped single elements, which stain intensely with osmic acid. Apparently the trypsin digestion has divested the lamellae of certain (protein) components which bind the lipoid particles together.

3. *Replica-adhesion preparations of the sheath surface layers.* Shadowed replica-adhesion preparations of freshly dissected single nerve fibres, fixed with osmium or formalin vapors, provide a convenient way of studying the surface layers of the fibre stripped off successively. The first layers usually show a compact granular membrane covered with longitudinally oriented bundles of fibrils, most of which exhibit the typical axial repeating period of collagen fibrils (Plate IX a). Confirming evidence that this layer corresponds to the neurilemma with adhering connective tissue is furnished by tangential, longitudinal and transverse sections of frozen and embedded nerves. Immediately below the neurilemma, and corresponding therefore to the external surface layers of the myelin component, a *characteristic scaly structure* is seen. (Plate IX b.) It consists of irregular flakes or lamellae, 100–200 Å thick, piled upon each other like roof shingles or fish scales. These flakes are different from the already described myelin lamellae only because of their rough surfaces and irregular, fragmented appearance, but otherwise they are quite similar even as regards the fine granular surface structure. It is possible that the scaly texture is produced by the employed stripping procedure, which tears off the superficial layers of the myelin lamellar structure in this irregular fashion. Continuation of the stripping procedure on the same fibre repeatedly shows this type of scaly structure until the characteristic fibrous layers of the axon have been reached. The round or oval bodies (0.5–1 μ in diameter), containing a dense granular substance, which have been described earlier in the vicinity of the Schwann cells (16), were encountered frequently in these preparations.

e. THE EXTERNAL MEMBRANE AND FIBROUS LAYERS OF THE SHEATH

The granular membrane with attached fibril bundles surrounding the myelin sheath and readily detached from it has already been described as corresponding to the neurilemma of classical terminology. It is one of the most conspicuous structures of the fibre, and so easy to study because it generally peels off as a thin intact membrane in frozen sections (Plates IX a, b) or in replica-adhesion preparations (Plate X a). The neurilemma consists of a uniform, approximately 200 Å thick compact granular membrane adhering closely to the myelin component and covering the examined internode portions of the fibre completely. (Tears in the membrane may have been produced during preparation.) The relation of the Schwann cell to the neurilemma has not been investigated, and only its general morphological features will be described here. In osmic acid fixed frozen sections which have not come in contact with lipoid solvents, the membrane appar-

ently consists of densely packed uniform granules of dimensions close to the resolving power of the microscope. After extraction of the unfixed, air-dried fibre with acetone, most of the granules disappear and only a transparent membrane with numerous holes and a faint granular structure is seen. (Plate X a.) The granules may therefore represent a lipoid containing structure. Two types of fibrils are found lying on top or attached to the membrane. Longitudinally arranged fibrils of variable width (500–700 Å), with a characteristic axial repeating period of an average of 600–660 Å, are the most common type found. Since this axial periodicity is in the range typical of collagen and in some cases more detailed intraperiod structure similar to the six banded pattern of collagen has been observed, *these fibrils are considered to be collagen fibrils.* The collagen fibrils are arranged predominantly in longitudinal bundles with the cross bands in nearly perfect register. The other type of fibril is not so frequently encountered and differs markedly from the collagen fibrils, since it is essentially *smooth*, showing no axial periodicity, generally *wider* (average width: 700–800 Å), *more dense*, and has a typical *rigid*, wirelike or coiled appearance. These smooth, rigid fibrils generally occur single, running contorted independent courses and sometimes interconnecting to form sharply outlined, wide meshed nets. Thin (100 Å) smooth fibrils bound together in compact strands are also found (17). These fibres have not been studied further, but it is possible that they are related to the elastic tissue elements responsible for the elasticity of Henle's sheath (de Rényi 9). The general arrangement of the fibril bundles ranges between a predominant longitudinal orientation or oblique interlacing, depending upon the stretched condition of the nerve fibre during fixation.

While examining formalin fixed frozen sections of rat and frog sciatic nerves, certain fibrils with dense edges and low density in the central axis, resembling the "neurotubules" described by de Robertis and Schmitt (11), were found distributed at random among the collagen fibrils of the external sheath layer. Closer examination revealed numerous transitions between solid, typical collagen fibrils and tubular appearing fibrils with dense edges and partially visible axial periodicity. It was noted that the tubular fibrils are embedded in a hazy material which obliterates the background detail and seems to come from the myelin sheath. In those areas which are not contaminated by this material the same fibrils stand out as "solid", characteristic collagen fibrils against a clear, granular film background. After observing the same conditions in all electron micrographs showing "neurotubules", it appeared possible that *the "neurotubules" are in reality collagen fibrils invested with the hazy substance.* This possibility was further supported

by the complete *absence of structures resembling the neurotubules in the axon* or in any other part of the fibre other than the external fibrous layer of the sheath, and here only in formalin fixed material. Bearing in mind the evidence given by de Robertis and Schmitt (11) "that the neurotubules are located in the axis cylinder of nerve fibers" these structures were particularly looked for in the axon, but never seen among the characteristic filaments found instead. Schmitt (37), in a re-examination of the origin of the neurotubules has found these dense-edged fibrils in the sheath of squid nerves from which the axoplasm had been removed. This would also indicate that the neurotubules are sheath components.

II. *Structures in the axon of rat sciatic nerve fibres*

Examination of osmium fixed, frozen transverse sections, 1–3 μ thick, shows that the space occupied by the axon is generally empty, but a certain number of sections reveal two fibrous structures within the lumen of the fibre. These are: (1) *A dense fibrous net or membrane lining the internal wall of the sheath,* (2) *A network of thin beaded fibrils, 100–200 Å in width, bridging the lumen of the axis cylinder.* Collodion-paraffin embedded sections preserve these fine axon structures much better, and the following descriptions are based on the combined information obtained by the two methods.

1) *The internal fibrous membrane* consists of interlaced fibrils, 100–200 Å wide, densely woven to form a continuous net coating the inner sheath wall and providing the basis for the fine reticulum in the axis cylinder. (Plate XI a.) Therefore, it would correspond to the postulated axolemma membrane, since it is located at the border between sheath and axon, enveloping the latter. That this net is a separate, constant structure can be clearly seen in transverse and longitudinal frozen sections in which the axon is empty and only the delicate, fibrous girdle partly detached from the inner wall has remained intact. In formalin and osmic acid fixed preparations the fibrils have a typical beaded or nodose appearance with indications of a certain periodicity, which is mainly of the order of 600–700 Å, although smaller periods of approximately 200 Å were resolved in some fibrils. The unit fibrils are generally bound together or intertwined to form compact bundles, associated with a diffuse granular substance. But the characteristic feature of this membrane is the large number of *dense, longitudinally oriented trabecular reinforcements.* (Plates X a, b.) These reinforcements resemble dense bands or fibers, 0.2–0.6 μ wide, arranged longitudinally and running independent courses over long extensions of the nerve fibre. Sometimes they

can even be made out in longitudinal sections with the light microscope, appearing as thin fibrils in the axon. Closer study of the micrograph shows that they are really *dense braids* of intertwined unit fibrils which are compactly bound together. The higher density results from this compact aggregation of fibrils and probably from the presence of a (cementing) substance which stains heavily with osmium. In transverse sections these trabecular reinforcements look like thick bands evenly distributed around the entire circumference of the thin fibrous membrane. The attachment of the axolemma membrane to the inner wall of the sheath seems to be more firm along these reinforcement bands. The general appearance of the net and specially the width and configuration of its meshes depends upon the state of tension to which the nerve fibre was subjected during fixation. Thus, in specially stretched fibres of the frog sciatic during osmic acid fixation, typical oblique interlacing of the axolemma fibril bundles and distension of the meshes reveal the stretched condition. (Plate XI a.) After alcohol-ether extraction of fresh nerve fibres some of the granular substance which coats the axolemma membrane, specially where it is in contact with the myelin sheath, is removed. The fibrils themselves are unaltered and frequently show fine structural details better than in ordinary, fixed preparations. Occasionally smooth, rigid fibrils resembling the ones described in the external sheath layers were found in the axolemma membrane, but since the possibility of sheath contamination could not be ruled out, further evidence is needed to support this finding.

2) *The network in the axis cylinder.* This network is seen both in osmic acid and formalin fixed frozen sections, and in collodion-paraffin embedded nerve, particularly well preserved in the latter. It consists of 100–200 Å wide, nodose filaments, stretched transversally or in an oblique transverse direction across the lumen of the nerve fibre. A wide meshed net is the result, which appears unusually empty in frozen sections, but is filled with a dense granular material in collodion-paraffin embedded sections. The filaments are very similar to the fibrils of the axolemma membrane, but the configuration of the axon reticulum does not exhibit the regular characteristics of the axolemma net, and is partially masked by the dense masses of adhering granular material. This is most marked in the central parts of the axon, where lighter areas of aggregated granules (50–200 Å in diameter) alternate with dense patches of material which sometimes assume the shape of larger fibers or long contorted rods. The smaller granules frequently adhere to the filaments of the reticulum, contributing to their nodose appearance. (Plates XII, XIII.) Both the reticulum and the granular substance within the axis cylinder are

not affected by treatment with acetone, amylacetate or ether after fixation, they also show up well after alcohol-ether extraction of fresh whole nerve. The axon reticulum is preserved only in certain thick frozen sections, where its wide, irregular meshes are mostly empty, and particularly well in collodion-paraffin embedded sections after alcohol dehydration. The observation that most of the thinner frozen sections merely show the partially detached axolemma net, while the reticulum has disappeared, might indicate its more fragile nature and loose connections with the sheath walls. To what extent this reticulum is a fixation product can only be determined by specially devised investigations, since the axis cylinder is one of the most sensitive components of the nerve fibre.

III. *Sheath and axon structures of the frog sciatic nerve*

The myelin sheath and axon structures described in rat sciatic nerve are essentially similar to the corresponding structures observed in frog sciatic fibres. The differences found are quantitative and subject to the limitations inherent in the accuracy of measurements. The myelin lamellae of the frog seem to be thinner than the lamellae of the rat sciatic (Table I.) The average spacing of the concentric lines superimposed on the laminated structure in thin sections is also of smaller magnitude. (Table II.) Minor differences in the configuration of the external and internal fibrous layers would concern a more detailed study which is not within the scope of this investigation.

IV. *The general features of the nerve fibre organization in the internode portion*

Since it is obviously impossible to present all of the material accumulated in the course of this study, which is based on examination of several hundred sections, an attempt has been made to give an integrated picture of the observed structural relations in the following schematic diagram. The diagram (Plate XIV) depicts an idealized internode portion of the myelinated nerve fibre, from which the Schwann cell and the possible structures of the Schmidt-Lantermann incisures have been left out. Therefore, in terms of conventional terminology, the diagram would correspond to a single cylindroconic segment. Essentially it represents a concentrically laminated tube invested with an external membrane, the neurilemma (N) with adjacent connective tissue, and containing a cylindrical core: the axon (A) which is enclosed in a fibrous net, the axolemma membrane (Ax). While the location and structural details

of these membranes and the fibrous reticulum of the axis cylinder (which is merely indicated in the sketch) could be established with reasonable certainty, the structure of the myelin component (M) as shown here was not observed directly with such clarity. It rests largely on the fact that the myelin sheath separates into concentrically arranged rings from which thin lamellae peel off, and on the more important observation of a concentric laminated structure in thin transverse and longitudinal sections of osmium fixed nerve. The isolation of similar lamellae from fragmented thin frozen sections in which the myelin represents the largest component, and the numerous transitions observed, showing the lamellae in the process of separation from the bulk of the sheath, further support this interpretation. Moreover, structures resembling these lamellae have been found in myelin droplets released from fragmented, fresh nerves, after fixation with osmic acid or formalin. The scaly lamellar structures described in replica-adhesion preparations of dissected nerve fibres could also be correlated with the proposed laminated structure of the sheath. Finally, the myelin structure sketched in the diagram is in general agreement with the results of polarized light analysis and X-ray diffraction studies. The schematic representation of a series of thin walled tubes fitted tightly into each other and extending unbroken throughout the sheath is however merely conjectural, since the details of the sheath structure have not been investigated well enough yet. Further improvements in sectioning technique are necessary before this problem can be studied. The crevices shown in the sheath are seen mainly in frozen sections, and if it can be shown that they really exist and are not desiccation artefacts, might represent liquid-filled interstices. It is interesting to recall in this connection that Schmitt, Bear and Palmer (42) already discussed the "existence of micelles or slabs of 'myelin' separated by relatively thick channels of aqueous phase", the latter possibly representing faults or crevices in the sheath.

Comparison of the details in the drawing with the corresponding electron micrographs will give a general idea of the fibre's organization as derived from these preliminary studies, but the tentative character of certain aspects of this diagram is emphasized.

H. DISCUSSION

The numerous artefact possibilities introduced by the fixation, sectioning, desiccation and electron bombardment inherent in the methods employed here will be considered first.

1. Fixation with osmic acid, used predominantly in this study, does not change the polarization optical picture of myelinated nerve significantly, as W. J. Schmidt (33) has shown. Although impregnation with osmic acid abolishes the birefringence of lipoids, the black reaction product of the osmic acid (osmium dioxyhydrate) is deposited in an oriented way in the myelin sheath, introducing a weak positive uniaxial birefringence with radially oriented axis, and thus partially compensating for the loss of the lipoid birefringence. Osmic acid is therefore particularly suitable as a fixative agent for nervous tissue because it preserves the original lipid structures to a certain extent, without disturbing the protein structures. The X-ray diffraction patterns of osmic acid treated nerve described by Schmitt, Bear and Clark (41) which were interpreted as indicating the "presence of large lipoidal aggregates probably in random fashion" are difficult to evaluate in this connection. The lamellar structures are not only shown after osmic acid fixation, but were also seen in formalin fixed material, after alcohol fixation and in airdried nerves.

2. As regards the artefacts which might be produced by the sectioning techniques employed:

a) By using frozen sections, embedding of the nerve fibres was avoided. This eliminates the prolonged treatment with alcohol, ether, collodion or plastic solutions as well as the paraffin embedding, which could give rise to a number of structural alterations due to extraction of lipids, permeation effects of the embedding medium, etc. No matter what embedding agent is used, subsequent examination with the electron microscope requires that it be removed from the specimen (because of the greater contrast, also for metal shadow casting). Complete removal of the embedding substance involves prolonged treatment with solvents, and this inevitably introduces the possibility of fine structural alterations. Even if a water soluble embedding medium like gelatine is used, it has been very difficult to remove the last disturbing traces of this material in the specimen. The minimum of manipulations required in the described serial frozen section technique is a further advantage.

b) On the other hand, distortion of the unembedded sections and possible disolocation of structures when cutting and mounting the specimens are typical disadvantages of frozen sections. This is of particular importance in the interpretation of the axon structures and the location of the sheath membranes. But here it was possible to check the findings by using embedded material (collodion-paraffin and gelatine embedding) and the modifications were generally found to be negligible. *A combination of both procedures is evidently the best solution of this problem.*

3. Drying of whole nerve does not seem to alter the submicroscopic organization of the nerve fibres considerably, judging from the X-ray diffraction patterns of dried nerve (41, 42). In the case of frozen sections, drying occurs in the last stages of preparation, when the section has already been cut and mounted on the supporting membrane. Comparison of fixed thin frozen sections with equally thin collodion-paraffin embedded sections reveals no significant differences between the two. *Drying often leads to separation and splitting along natural cleavage planes, but it does not produce artificial demarcations.* This is important in evaluating the laminated structure of the myelin sheath and its cleavage into leaflets described in dried frozen sections, which has its equivalent in the well known tendency of the sheath to separate out in concentric lamellae under ordinary (wet) conditions. (Nageotte, 25). Elec-

tron bombardment does not visibly affect well fixed specimens. It was generally kept at a minimum by using low electron optical magnification and the telescopic image viewing device.

After this general analysis of possible artefact sources, the electron microscope findings can be discussed in the light of the available information on the ultrastructure of the sheath. The interpretation of the polarized light and X-ray diffraction studies given by Schmitt, Bear and Palmer (42) would lead us to expect a laminar structure consisting of lipid-protein layers approximately 180 Å thick wrapped concentrically about the axon. Thin sections of osmic acid fixed nerve show the presence of a concentric laminated structure throughout the sheath wall, consisting of *individual layers which are only 80 Å thick*, approximately. It is assumed that these layers are formed by thin lamellae arranged concentrically, since transverse sections separate into concentric rings from which the lamellae peel off. Also because piles consisting of unit lamellae with an average thickness of 80 Å are obtained by fragmenting thick frozen sections. The variable height and dimensions of the lamellar surface structures seen in osmic acid fixed specimens might be due to artificial aggregation of the normally evenly distributed material. The term "wrapped" is avoided at present because it might implicate the continuity of a single membrane wrapped many times around the axon to form the concentric layers. The simpler arrangement of individual thin walled tubes or rings fitted tightly and concentrically into each other was tentatively suggested in the diagram.

According to the interpretation of Schmitt, Bear and Palmer (42) the layers of lipids alternate with thin sheets of protein, estimated to have a thickness of 25–30 Å. Thin sections of whole nerve extracted with alcohol-ether and subsequently fixed in osmic acid show only smooth lamellae, 30–40 Å thick, devoid of the granular surface structure seen in osmium fixed lamellae of unextracted nerve. This may indicate that the myelin lamellae consist of a stable protein film, 30–40 Å thick, covered with a granular surface structure 60–120 Å high, which presumably represents the lipid component, since it is removed by alcohol-ether extraction. The latter would correspond to one or two double layers of lipids (Bear, Palmer and Schmitt (6) found a period of 63.7 Å for one double layer of a dried total mixture of nerve lipids). The thickness of the rat myelin lamellae measured in shadowed preparations and the determination of the single layer thickness in thin sections show the same value of approximately 80 Å, which would correspond to only half the fundamental X-ray diffraction spacing of 158 Å found for mammalian

medullated nerve (42). In order to explain why the long spacing identity period corresponds to the thickness of two layers in the concentric laminated structure revealed by electron microscopy, we would have to consider the possibility of protein lamellae occurring between each double layer of lipids (42), resulting in a structure consisting of thin sheets of protein covered on both sides with lipid double layers. The granular fragmentation of the myelin lamellae obtained after prolonged incubation of whole fresh nerves in crude trypsin is more difficult to interpret, but might be explained by assuming that the protein basal membrane or some supporting substance of protein nature in the lamellae has been removed by the enzyme. The strikingly regular system of concentric lines superimposed on the concentric lamination and observed consistently in longitudinal and transverse osmic acid fixed frozen sections, must first be proven to derive from a real underlying structural regularity of the sheath and not to represent a spurious phenomenon. It is interesting to note, however, that the average line spacing of 70–80 Å measured in rat sciatic sections corresponds to approximately half the value of the fundamental X-ray spacing of 158 Å demonstrated in dried mammalian medullated nerve. The line spacing of 60–70 Å found in frog sciatic also corresponds to half the value of the 144 Å X-ray spacing obtained from dried frog spinal roots and sciatics (42). The general features of the sheath structure as revealed by electron microscopy seem to agree well with the results of polarized light and X-ray studies. The myelin lamellae have also certain features in common with the unit discs of the outer segments of guinea pig retinal rods as Sjöstrand (44) already pointed out. Sjöstrand (43) demonstrated that the outer segments of the retinal rods fixed in osmic acid break up into unit rounded discs with the same diameter as the outer segment. The unit discs consist of an inner membrane 30 Å thick with a cord-like structure, 70–80 Å thick, running along the edge.

Regarding the fibrous structures revealed in the axon the following points can be discussed:

(a) The network of nodose filaments, 100–200 Å thick, demonstrated in the axis cylinder of frog and rat sciatic nerve fibres probably corresponds to the reticulum of nodose fibrils, 200–400 Å in width, described in formalin fixed axons of Loligo and Myxicola giant fibers by Schmitt (37). The fibrous protein obtained from fresh squid axoplasm, which Schmitt (37) regards as the chief fibrous component of the axon, "yields fibrous structures ranging from relatively thick, rod-like bundles to very thin filaments". The question can be debated whether the fibrous reticulum shown in fixed sections is not the result of colloidal alterations induced by the fixative

which precipitates this fibrous component. Only further investigation can clarify this point.

(b) The net or axolemma membrane enveloping the axis cylinder seems to represent a constant structure, and its longitudinally oriented trabecular reinforcements are too well defined to be a fixation product. The presence of this axolemma membrane would partly explain why a naked axis cylinder stripped of its myelin sheath keeps its shape (de Rényi 9). The droplets of myelin left behind when this component is pulled off (de Rényi 10) might be located in areas of more firm attachment between axolemma and myelin sheath. It would be premature to discuss the polarized light studies on the fresh axon (5) until more information on the axon structures is available. The determination of mass in the axon of single nerve fibers using the X-ray absorption technique by Engström and Lüthy (13) leads to the discussion of two possibilities for the arrangement of submicroscopic fibrils in the axon, One of these, the assumption of solid submicroscopic fibrils in the axon, is in agreement with the electron microscope findings. Correlation of the neurofibrils with any of the structures described in the axon is not possible at present. The longitudinal reinforcements of the axolemma, appearing constantly in osmic acid fixed preparations, have diameters up to 0.6 μ and could therefore approach the dimensions of finest neurofibrils, but they are mainly confined to the peripheral parts of the axon. Aggregation of the fine axon filaments, accompanied by deposition of the diffuse granular substance, may give rise to the longitudinal fibrils seen in the interior of the axon, specially in collodion-paraffin embedded sections. Whether these correspond to the neurofibrils can only be determined by comparing electron and light microscope pictures of appropriately stained sections.

Now that thin sections can be examined with the electron microscope it is hoped that progress in the structural analysis of nerve fibres will be rapid, and that further studies may eventually disclose the key structures involved in nerve impulse propagation.

SUMMARY

Ultrathin serial frozen sections of osmic acid and formalin fixed frog and rat sciatic nerves, prepared with a described modification of the thermal expansion sectioning technique, were examined with the electron microscope. The study was supplemented by examination of thin sections of collodion-paraffin, gelatine embedded nerve and replica-adhesion preparations of

single nerve fibres. The following structures are described in the internode portion of the rat and frog nerve fibres:

1. (a) The myelin sheath is covered with an external fibrous layer consisting of a compact granular membrane, approximately 200 Å thick, which corresponds to the neurilemma. Longitudinally arranged bundles of collagen fibrils and a smooth, rigid type of fiber are attached to this membrane. The dense-edged fibrils which had been described as "neurotubules" are collagen fibrils of the external sheath layers invested with a dense, hazy substance.

(b) The main part of the myelin sheath separates into concentric rings from which thin lamellae peel off. These lamellae are identical with the flexible lamellar structures isolated single or in piles from fragmented frozen sections. The unit lamellae are 50 Å thick, with a variable granular surface structure 50–100 Å high. The sheath exhibits a concentric laminated fine structure in thin transverse and longitudinal sections, and the individual layers of this structure are approximately 80 Å thick. A concentric system of extremely regular and uniform lines, superimposed upon the concentric lamination of the sheath, with an average line-spacing of 70–80 Å, was seen consistently in transverse and longitudinal, osmic acid fixed frozen sections. After alcohol-ether extraction of whole nerve thin, concentrically arranged leaflets, 30–40 Å thick, can be demonstrated, which are similar to the myelin lamellae devoid of granular surface structure and may be protein films. Lamellar structures which disintegrate into rod-shaped granules, 50–100 Å high, are described in nerves incubated with trypsin. Structures resembling the lamellae of fixed nerve were found in osmium and formalin fixed myelin droplets released from fragmented, fresh nerve fibres. A scaly surface structure, corresponding to the outer myelin layers of the sheath, was observed in replica-adhesion preparations of single nerve fibres. Based on these observations a general concentric laminated structure of the myelin sheath is assumed, and the details of this structure compared with the results of polarized light and X-ray diffraction studies.

2. (a) A dense net of interlaced nodose fibrils, 100–200 Å in width, with longitudinal trabecular reinforcements, coats the internal sheath wall and provides the basis for the fine reticulum in the axis cylinder. It probably corresponds to the axolemma membrane.

(b) Thin nodose filaments, 100–200 Å in width and with certain indications of an axial periodicity, are found in the axis cylinder, extending in a transverse or oblique longitudinal direction, and forming a network which is filled with a granular substance present in different states of aggregation.

ACKNOWLEDGEMENTS

Part of the expenses for this investigation were defrayed from research grants by the Wallenberg Foundation, the Therese and Johan Anderson foundation, and by the Venezuelan Government. I wish to express my sincere thanks to Dr. A. Martin Araujo, Venezuelan Public Health Minister, and to Dr. A. Mijares, Venezuelan Minister of Education, for their constant interest in the course of this work. It is a pleasure to thank Professor T. Caspersson for his kind help and for placing all the facilities of his institute at my disposal. My sincere thanks are due to Miss L. Holmqvist for her valuable technical assistance. I would also like to thank Prof. R. Lucchetti for the excellent diagram of the fibre; Mr. H. Walén, L. Wegstedt, L. Aquilonius, R. Säfström and all the members of the Department of Cell Research, Karolinska Institutet, for their generous help.[1]

REFERENCES

1. Ambronn, H., *Berichte sächs. Ges. d. Wiss. Math.-phys. Cl.*, **42**, 419 (1890).
2. —— *Kolloidzschr.*, **6**, 1 (1910).
3. Auerbach, L., *Pflüg. Arch. ges. Physiol.*, **222**, 493 (1929).
4. Bear, R. S., and Schmitt, F. O., *J. cell. comp. Physiol.*, **9**, 275 (1937).
5. Bear, R. S., Schmitt, F. O., and Young, J. Z., *Proc. roy. Soc.* B, **123**, 496 (1937).
6. Bear, R. S., Palmer, K. J., and Schmitt, F. O., *J. cell. comp. Physiol.*, **17**, 355 (1941).
7. Boehm, G., *Koll. Ztschr.*, **62**, 22 (1933).
8. Chinn, P., and Schmitt, F. O., *J. cell. comp. Physiol.*, **9**, 228 (1937).
9. De Renyi, G. St., *J. comp. Neurol.*, **48**, 293 (1929).
10. —— *J. comp. Neurol.*, **48**, 441 (1929).
11. De Robertis, E., and Schmitt, F. O., *J. cell. comp. Physiol.*, **31**, I (1948).
12. Ehrenberg, Ch. G., *Monatsber. d. preuss. Akad. d. Wissensch.*, 64 (1849).
13. Engström, A., and Lüthy, H., *Exper. Cell Res.*, **1**, 81 (1950).
14. Ettisch, A., and Jochims, J., *Pflüg. Arch. ges. Physiol.*, **215**, 519 (1926).
15. —— *Pflüg. Arch. ges. Physiol.*, **215**, 525 (1927).
16. Fernández-Morán, H., *Proc. Electr. Micr. Meet. Cambridge*, Sept. 20 (1948).
17. —— *Exper. Cell Res.*, **1**, 141 (1950).
18. Fernández-Morán, H., and Luft, R., *Acta Endocrinol.* II, **3**, 199 (1949).
19. Göthlin, G. F., *K. svenska Vetensk. Akad. Handl.*, **51**, 1 (1913).
20. Handovsky, H., *Koll. Ztschr.*, **62**, 21 (1933).
21. Hillier, J., and Ramberg, E. G., *J. Appl. Physics*, **18**, 48 (1947).
22. Massazza, A., *Arch. ital. Anat. Embriol.*, **26**, 89 (1928).
23. Mezzino, L., *Riv. Biol.*, **13**, 31 (1931).
24. Muralt, A. v., *Die Signalübermittlung im Nerven*, Birkhäuser, Basel (1946).
25. Nageotte, J., *Arch. mikr. Anat.*, **77**, 245 (1911).
26. Newman, S. B., Borysko, E., and Swerdlow, M., *Science*, **110**, 66 (1949).
27. Palmer, K. J., and Schmitt, F. O., *J. cell. comp. Physiol.*, **17**, 385 (1941).
28. Pease, D. C., and Baker, R. F., *Proc. Soc. exp. Biol. Med.*, **67**, 470 (1948).

[1] After completion of this manuscript the author has received a personal communication from Dr. F. O. Schmitt (dated Jan. 29th/50) in which he refers to a paper on thin nerve sections by Dr. Geren and F. O. Schmitt currently in press (J. Exp. Med.). In this paper structures similar to the nodose filaments of the axon of Loligo and Myxicola giant fibers (37) are described in thin sections of squid, frog, rat and human nerves. It is also proven that the "neurotubules" are sheath components.

29. Ramon y Cajal, S., *Textura del sistema nervioso*, Vol. I., Madrid (1899).
30. Richards, A. G., Steinbach, H. B., and Anderson, T. F., *J. cell. comp. Physiol.*, **21**, 129 (1943).
31. Schmidt, W. J., *Die Bausteine des Tierkörpers in polarisiertem Licht.* Bonn, 1924.
32. —— *Z. wiss. Mikr.*, **41**, 29 (1924).
33. —— *Z. Zellforsch.*, **23**, 657 (1936).
34. —— *Z. wiss. Mikr.*, **54**, 159 (1937).
35. Schmitt, F. O., *Cold Spring Harbor Symposia on Quant. Biology*, **4**, 7 (1936).
36. —— *Biochimica et Biophysica Acta*, **4**, 68 (1950).
37. —— *Progr. Ann. Meeting Electr. Micr. Soc. Amer.*, Oct. 8 (1949).
38. Schmitt, F. O., and Bear, R. S., *J. cell. comp. Physiol.*, **9**, 261 (1937).
39. Schmitt, F. O., and Bear, R. S., *Biol. Rev.*, **14**, 27 (1939).
40. Schmitt, F. O., and Palmer, K. J., *Cold Spring Harbor Symp. Quant. Biol.* **8**, 94 (1940).
41. Schmitt, F. O., Bear, R. S., and Clark, G. L., *Radiology*, **25**, 131 (1935).
42. Schmitt, F. O., Bear, R. S., and Palmer, K. J., *J. cell. comp. Physiol.*, **18**, 31 (1941).
43. Sjöstrand, F. S., *J. cell. comp. Physiol.*, **33**, 383 (1949).
44. —— *Proc. Conf. Electr. Micr. Delft.*, July 7 (1949).
45. Valentin, G., *Die Untersuchung d. Pflanzen und d. Tiergewebe in polarisiertem Lichte*, Leipzig, 1861.
46. Wolf, J., *Ztschr. wiss. Mikr.*, **56**, 57 (1939).

TEXTS TO THE PLATES

PLATE I a. Thick (2 μ) transverse frozen section of rat sciatic nerve fibre fixed in 1 per cent osmic acid. The internal fibrous membrane (a) lies, partially detached from the inner sheath wall, in the lumen of the fibre, while a bundle of typical collagen fibrils ("c") is shown attached to the outer part of the sheath. Platinum-palladium shadowed. Magnification: 16,000 ×, indicated by the drawn line which corresponds to one micron unless another scale is introduced.

PLATE I b. Transverse frozen section of rat sciatic fibre, osmium fixed. The myelin sheath separates into concentric rings from which thin lamellar ribbons (arrow "l") peel off. Notice bundle of collagen fibrils ("c") adhering to the outer sheath border. Pt/Pd shadowed. Magnification: 14,000 ×.

PLATE II a. Fragments of myelin lamellae from transverse frozen section of osmium fixed rat sciatic. The single layers or unit lamellae (approximately 50 Å thick), with peculiar droplet surface structures, are shown spread out fan-like and piled on top of each other. Pt/Pd shadowed. Magnification: 36,000 ×.

PLATE II b. Pile of myelin lamellae from transverse frozen section of osmium fixed rat sciatic. 5 lamellar layers with sharp, angular edges can be discerned in this large fragment. Pt/Pd shadowed. Magnification: 30,000 ×.

PLATE III a. Myelin lamellae isolated by gentle fragmentation from a longitudinal frozen section of osmium fixed rat sciatic nerve. Numerous folds in the lamellae and a fine granular surface structure can be seen. Pt/Pd shadowed. Magnification: 28,000 ×.

PLATE III b. Lamellar structure partially disintegrated into granules (50–100 Å high) isolated from a rat sciatic nerve incubated with crude typsin and subsequently fixed in 1 per cent osmic acid. Pt/Pd shadowed. Magnification 45,000 ×.

PLATE IV a. Thin (less than 0.4 μ) transverse frozen section of a rat sciatic nerve fibre fixed in osmic acid. The onion-peel-like separation of the myelin sheath into several concentric rings is clearly seen. Notice thin lamellar bands (arrow) pared off from the rings. Pd/Pt shadowed. Magnification: 16,000 ×.

PLATE IV b. Longitudinal frozen section through a nerve fibre of the rat sciatic. Osmic acid fixed, unshadowed. The edges (arrow) of at least 3 layers of myelin lamellae, which seem to be wrapped concentrically around the axon, can be distinguished. Magnification: 15,000 ×.

PLATE V a. Segment of the myelin sheath from a thin (approximately 0.1 μ) frozen transverse section of a rat sciatic fibre fixed in 1 per cent osmic acid. The characteristic concentric laminated structure of the myelin sheath is partially obliterated by the granular appearance of the single layers, but can be made out in certain parts. Notice the irregular oblong crevices between the layers. Pt/Pd shadowed. Magnification: 80,000 ×.

PLATE V b. Myelin sheath segment from an ultrathin frozen longitudinal section of osmium fixed rat sciatic. Six clear cut single layers of the concentric laminated sheath structure, each one approximately 70 Å thick, can be seen. Pt/Pd shadowed. Magnification: 160,000 ×.

PLATE VI a. Myelin sheath segment from an ultrathin frozen transverse section of osmium fixed rat sciatic. Several parallel layers (arrow) which follow the contours of a crevice in the sheath can be seen. Pt/Pd shadowed. Magnification: 80,000 ×.

PLATE VI b. Myelin sheath segment from an ultrathin frozen transverse section of osmium fixed rat sciatic in which it is difficult to distinguish between the characteristic sheath lamination (arrow) and the superimposed system of distinct, regular lines resembling a grating. Assuming that the lines actually correspond to the layers, an average thickness of 80 Å is found here. Pt/Pd shadowed. Magnification: 200,000 ×.

PLATE VII a. Myelin sheath segment from an ultrathin longitudinal frozen section of rat sciatic in osmic acid. The arrow points to the regular system of parallel lines bending around a crevice and which might correspond to the concentrically arranged sheath laminae. Pt/Pd shadowed. Magnification: 80,000 ×.

PLATE VII b. Myelin sheath segment from an ultrathin longitudinal frozen section of osmium fixed rat sciatic, exhibiting the extremely regular system of parallel lines referred to as the "grating effect" constantly seen in these sections. The line spacing measured here is approximately 68 Å. Pt/Pd shadowed. Magnification 150,000 ×.

PLATE VII c. Myelin sheath segment from an area adjacent to the one shown in Plate VII b, demonstrating the same regular system of lines bending around a crevice. Magnification: 190,000 ×.

PLATE VIII a. Myelin sheath segment from an ultrathin longitudinal frozen section of rat sciatic fixed in osmic acid, showing approximately 25 consecutive, relatively sharp, wavy lines, running parallel to each other and longitudinally oriented in the sheath. Pt/Pd shadowed. Magnification: 130,000 ×.

PLATE VIII b. Area adjacent to myelin sheath segment shown in Plate VIII a, demonstrating how the lines follow the contours of a crevice. Magnification: 200,000 ×.

PLATE IX a. External sheath membrane (neurilemma) with interlaced collagen fibrils, which has been separated from the sheath in a tangential section. Rat sciatic, osmic acid fixation. Pt/Pd shadowed. Magnification: 12,000 ×.

PLATE IX b. Longitudinal frozen section of a frog sciatic nerve fibre fixed in osmic acid. The neurilemma membrane with longitudinally oriented bundles of collagen fibrils (c) and single smooth, rigid fibrils (e) is seen. Pt/Pd shadowed. Magnification: 15,000 ×.

PLATE X a. Replica-adhesion preparation of a single, air-dried rat sciatic nerve fibre, extracted repeatedly with acetone, unshadowed. Notice the smooth dense fibril (arrow) running across the longitudinal bundles of fibrils exhibiting the characteristic axial periodicity of collagen. Remnants of the neurilemma membrane are visible as a transparent, granular film. Magnification: 16,000 ×.

PLATE X b. Replica-adhesion preparation of a single rat sciatic fibre fixed with osmium vapor and lightly shadowed with platinum/palladium. The scaly surface structure of the outer myelin layers of the sheath is shown. Magnification: 20,000 ×.

PLATE XI a. Longitudinal frozen section through a single fibre of frog sciatic nerve slightly stretched during fixation in 1 per cent osmic acid. The internal fibrous net with distended, narrow meshes and the characteristic longitudinal trabecular reinforcements (arrow), is shown. Closer examination will reveal that the trabecular reinforcements are formed by dense braids of the single fibrils. Pt/Pd shadowed. Magnification: 18,000 ×.

PLATE XI b. Longitudinal frozen section through a single fibre of frog sciatic nerve fixed in 1 per cent osmic acid. The meshes of the internal fibrous net are wider in certain areas, demonstrating the single fibrils (approximately 100 Å in width) with indications of a nodose periodic structure. Unshadowed. Magnification: 24,000 ×.

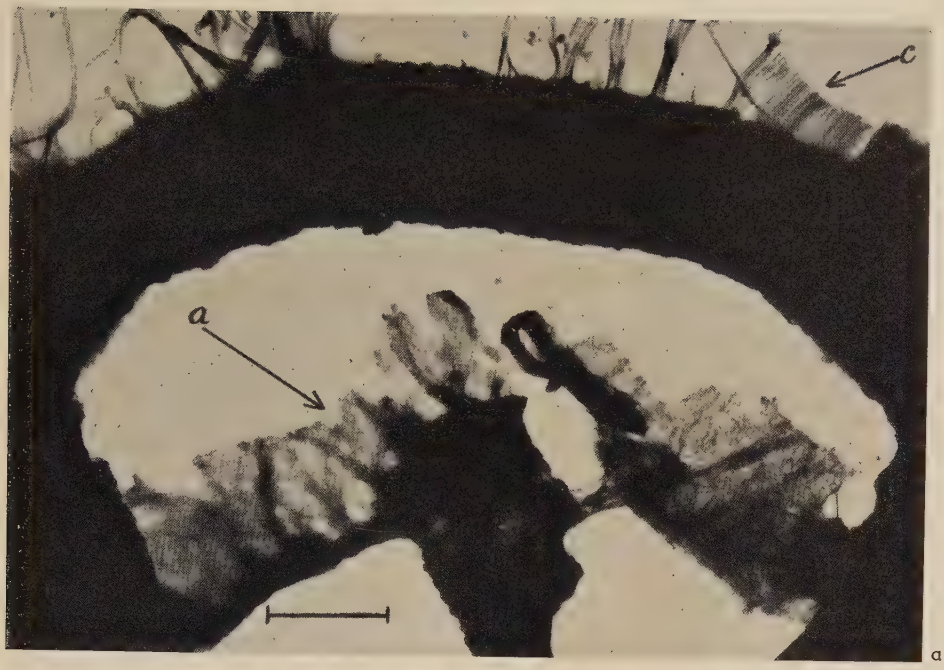
PLATE XII a. Transverse frozen section of rat sciatic nerve fibre fixed in 1 per cent osmic acid, showing the reticulum of nodose filaments stretched transversally across the axon. Pt/Pd shadowed. Magnification: 18,000 ×.

PLATE XII b. Transverse section of a fibre from rat sciatic nerve embedded in collodion-paraffin after alcohol dehydration. The fibrous reticulum is clearly visible only at the periphery of the axon, while the center is filled with a dense granular substance. Magnification: 16,000 ×.

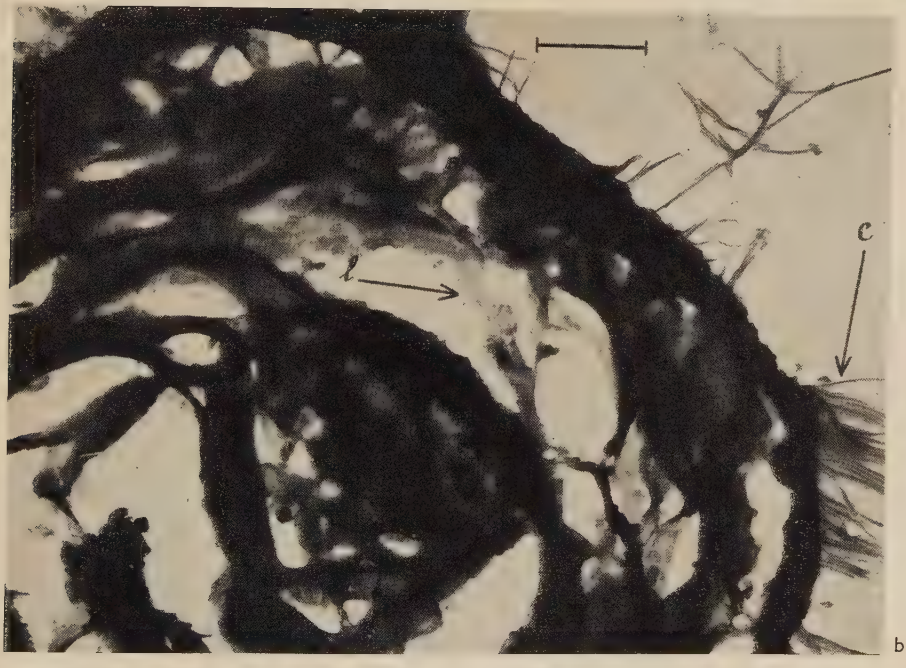
Plate XIII a. Transverse frozen section of a rat sciatic nerve fibre, showing the cob-web like axon reticulum with wide, empty meshes and numerous granules adhering to the fibrils. Unshadowed. Magnification: 18,000 ×.

Plate XIII b. Transverse section of a fibre from a rat sciatic nerve embedded in collodion-paraffin alcohol dehydration, demonstrating the reticulum and the dense granular material in the axis cylinder. Magnification: 19,000 ×.

Plate XIV:. Schematic diagram of the internode portion (corresponding to a cylindroconic segment) of a medullated nerve fibre to illustrate the general features and interrelationship of the structures described. The neurilemma membrane (N), with attached collagen fibril bundles (c) and smooth, rigid fibrils (e), (see Plates IX a, b and X a), envelops the scaly surface structure (Plate X b) corresponding to the outer, broken, layers of the myelin sheath (M). The latter is represented schematically by a series of (50) thin walled tubes, fitted tightly and concentrically into each other, and separated by irregular, oval crevices distributed at random throughout the sheath. The concentrically arranged lamellae are cut away to give the impression of the lamellae piles shown in Plates II a, b, III a, with indications of their droplet or granular surface structure. The internal fibrous membrane, probably corresponding to the axolemma (Ax) is depicted with its longitudinal trabecular reinforcements, while the axon reticulum is merely outlined. Magnification of the drawing indicated by scale of 1 micron length. Drawing by Prof. R. Lucchetti.

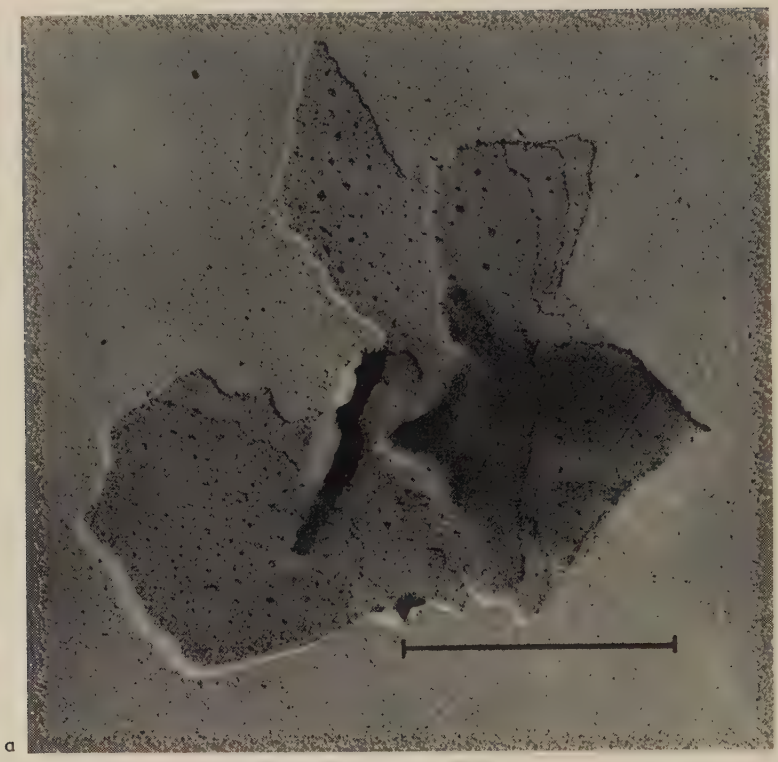


a

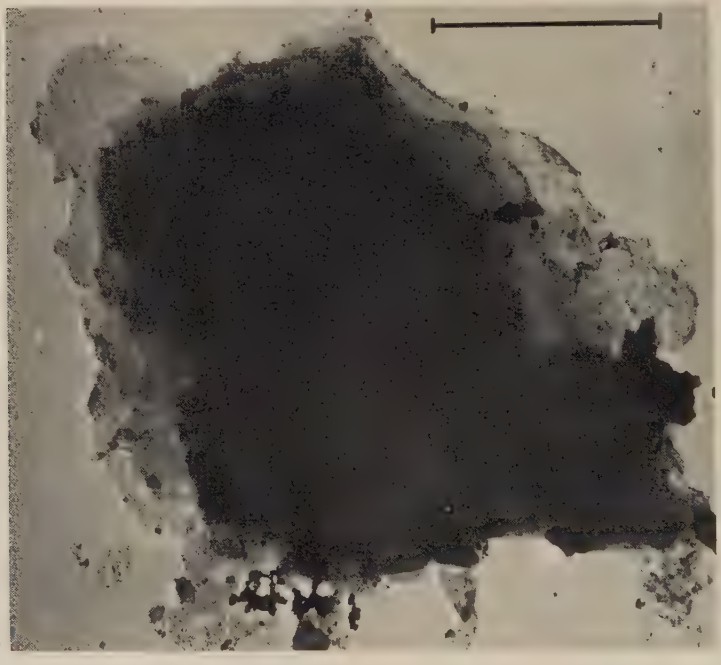


b

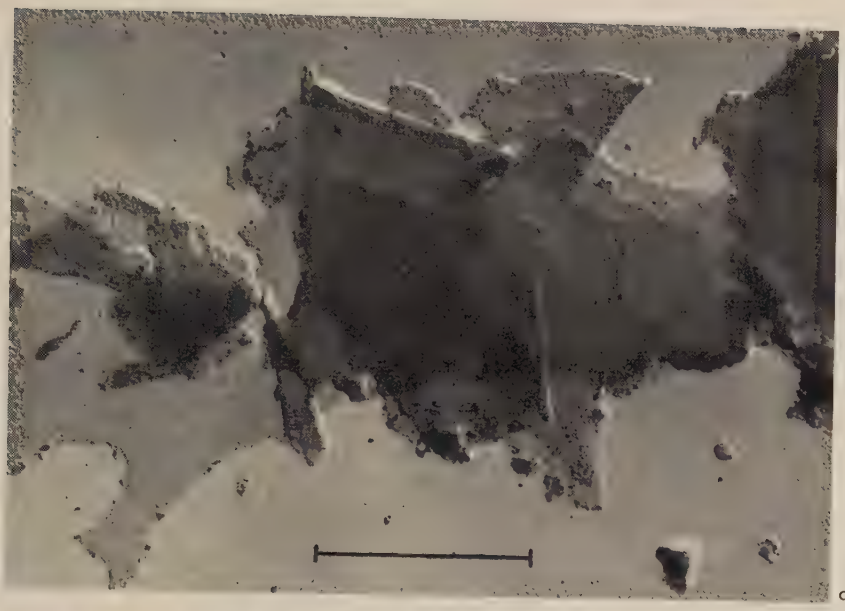
PLATE II



a

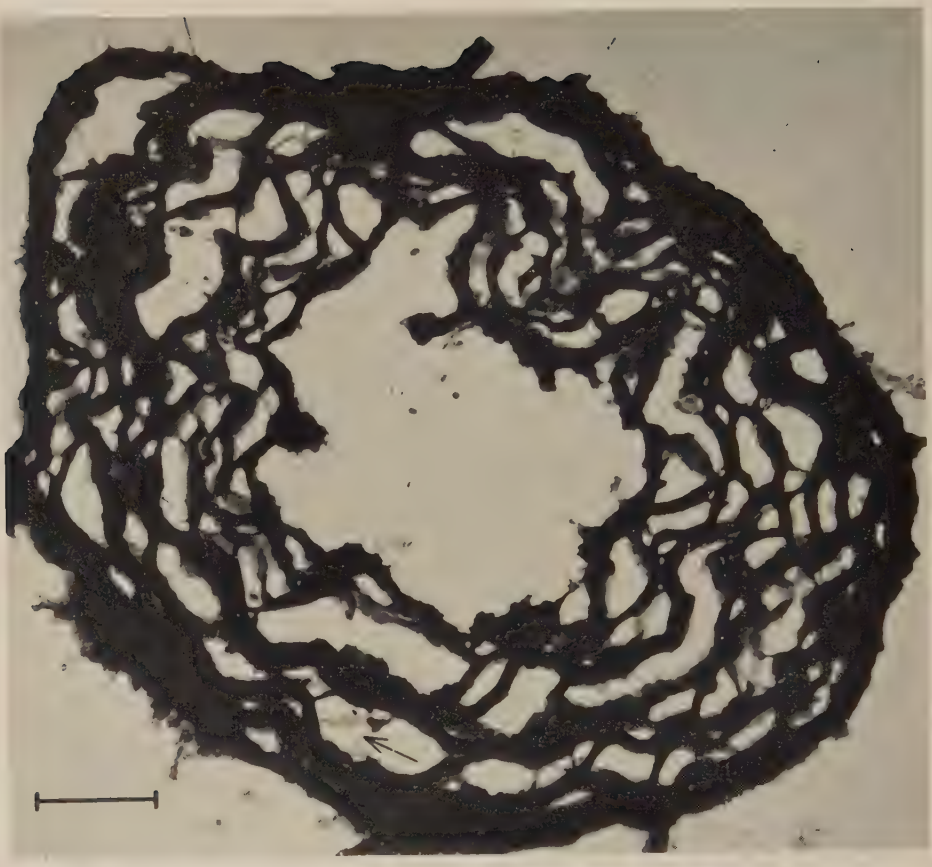


b

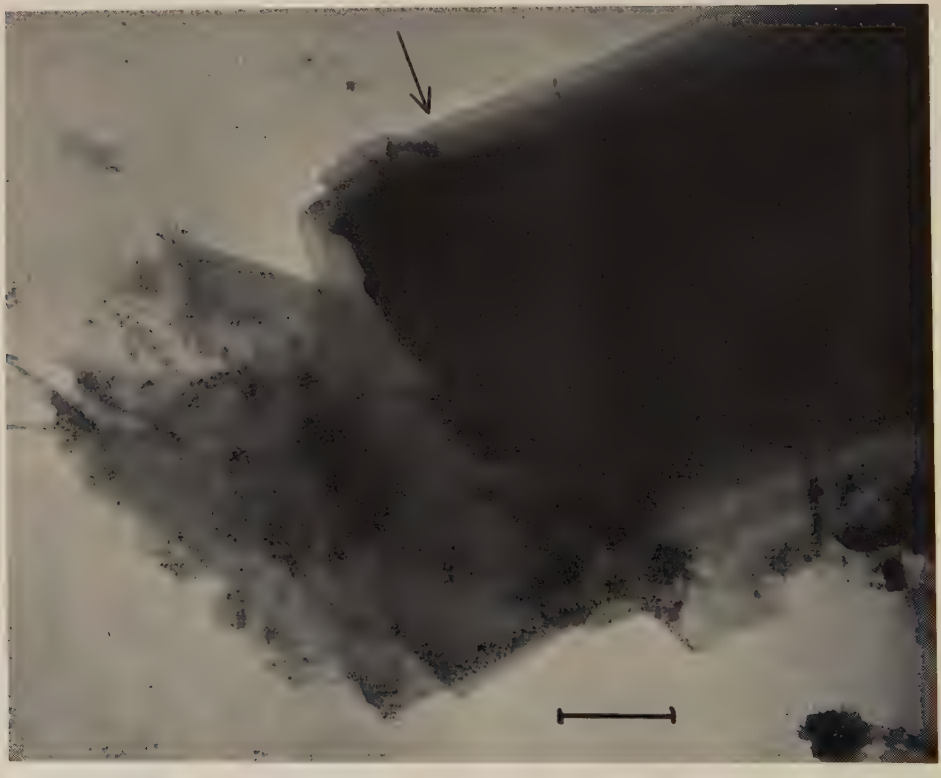


a

b

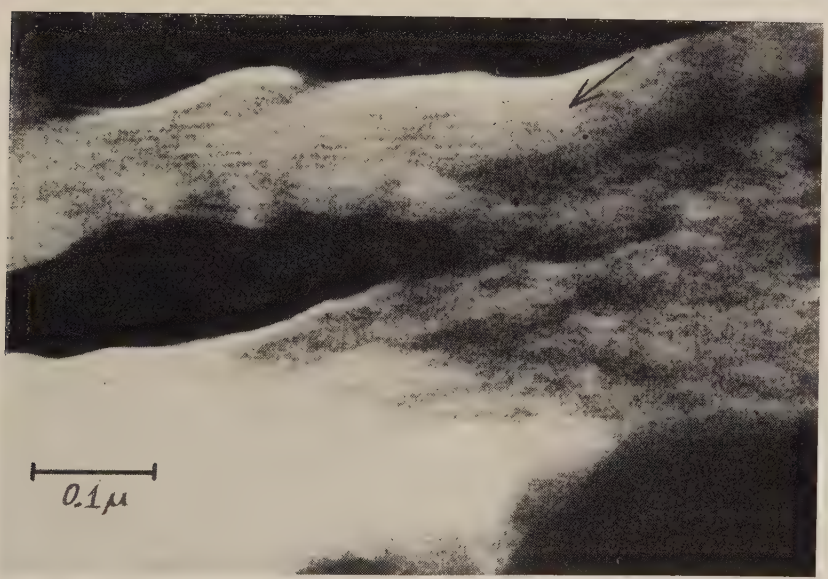


a

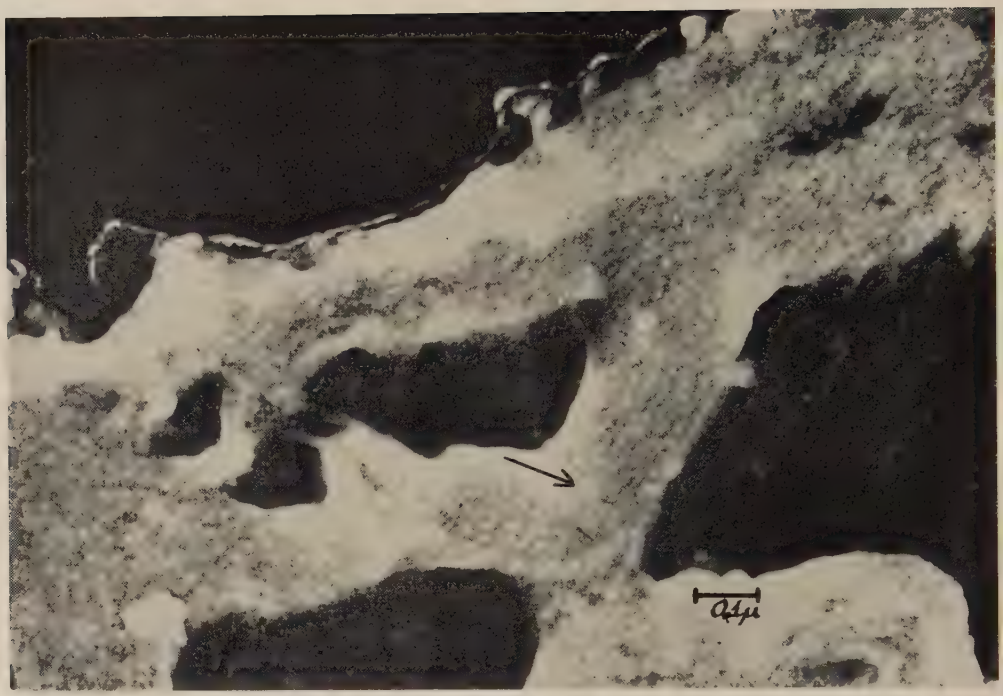


b

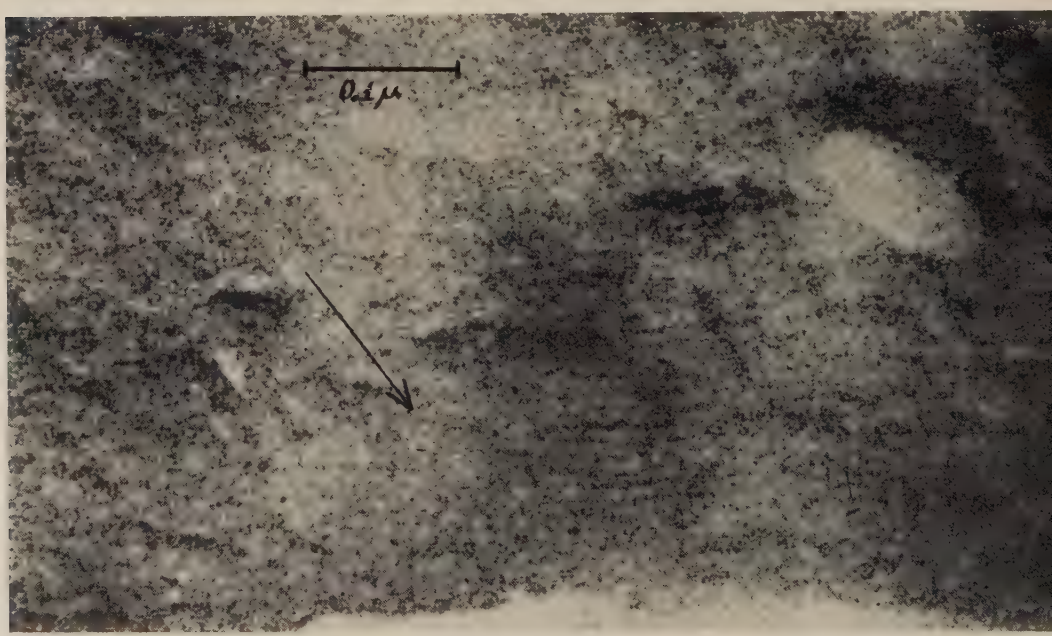
a



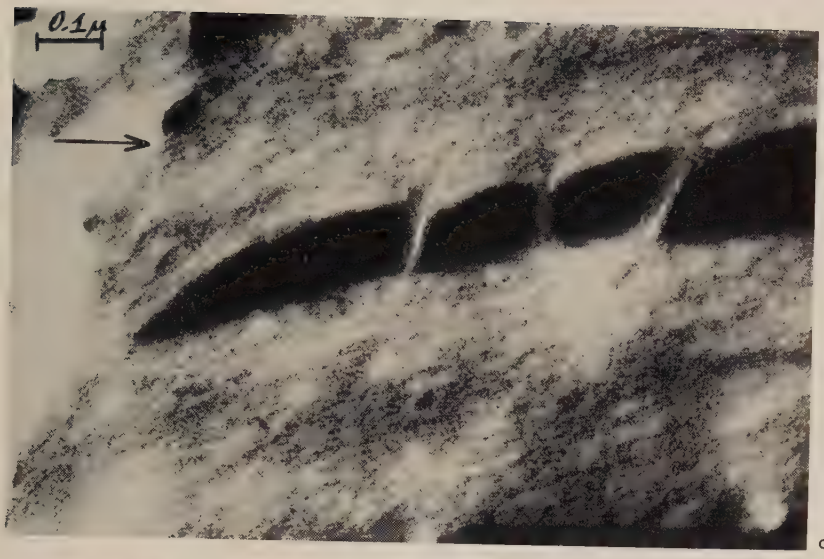
b



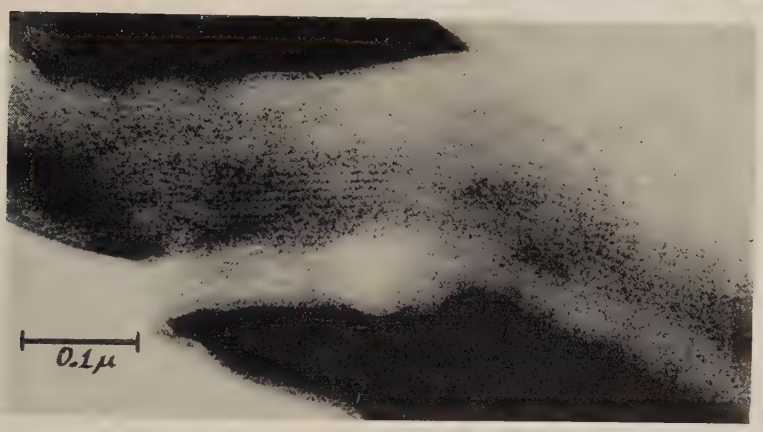
a



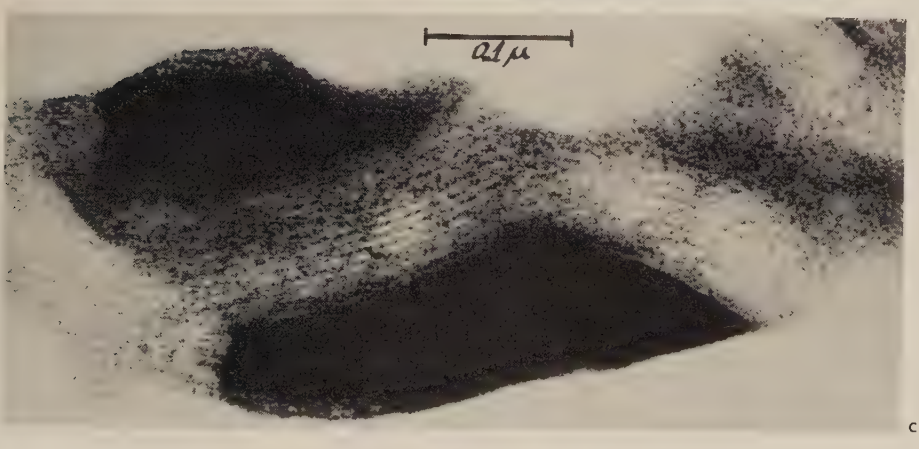
b



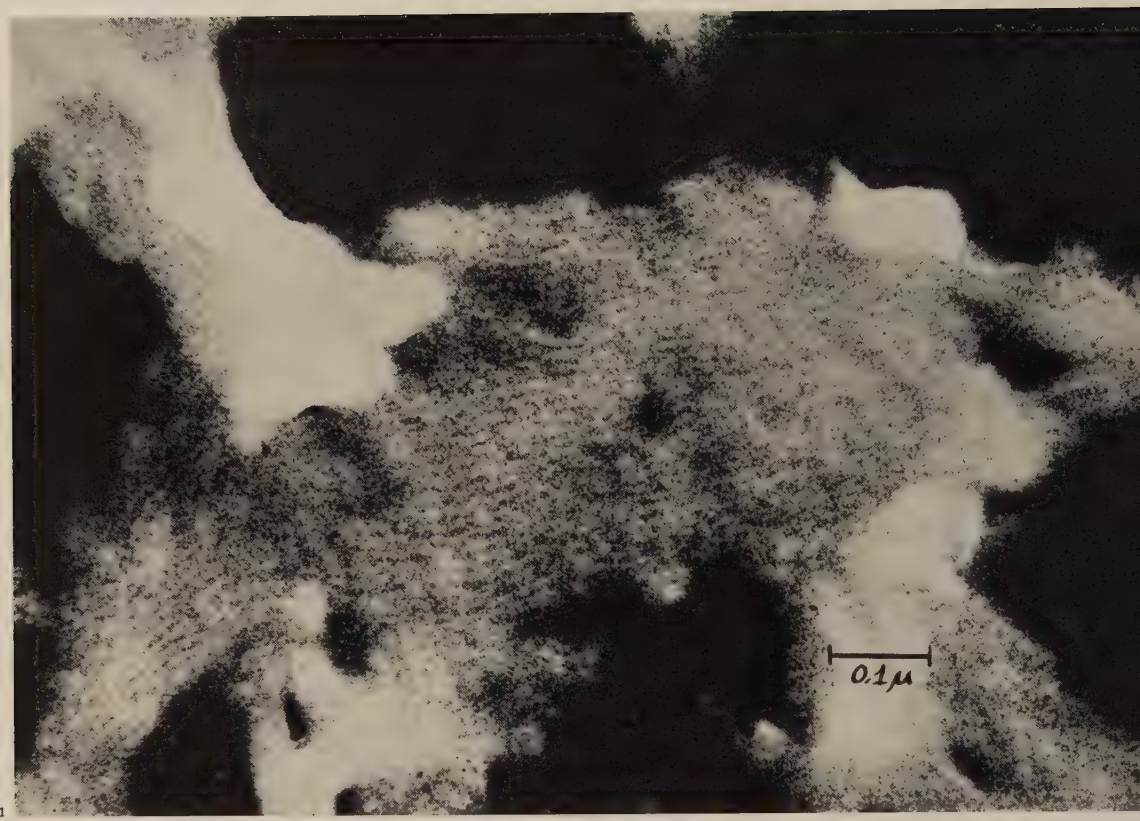
a



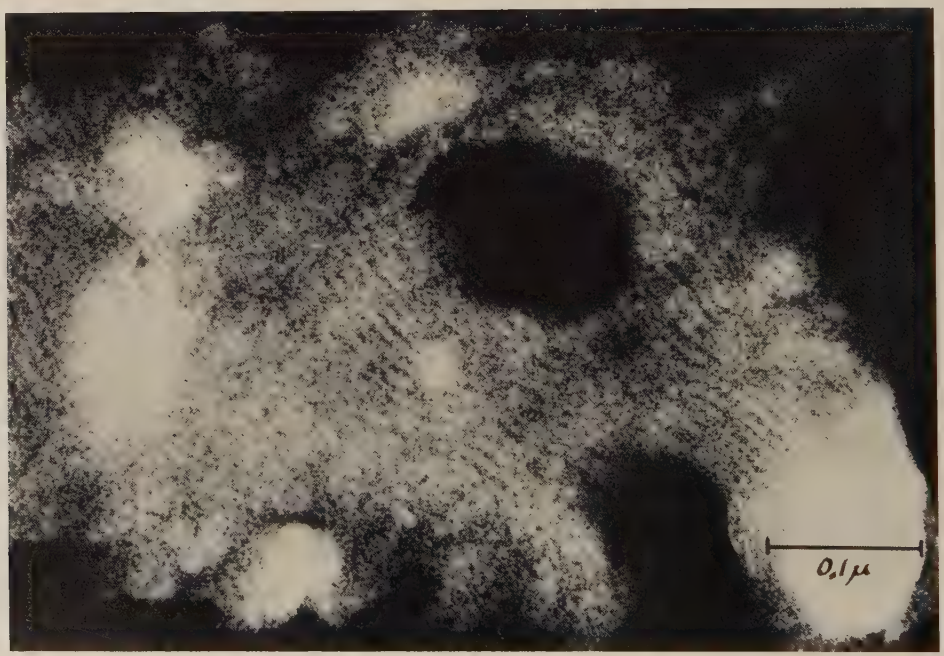
b



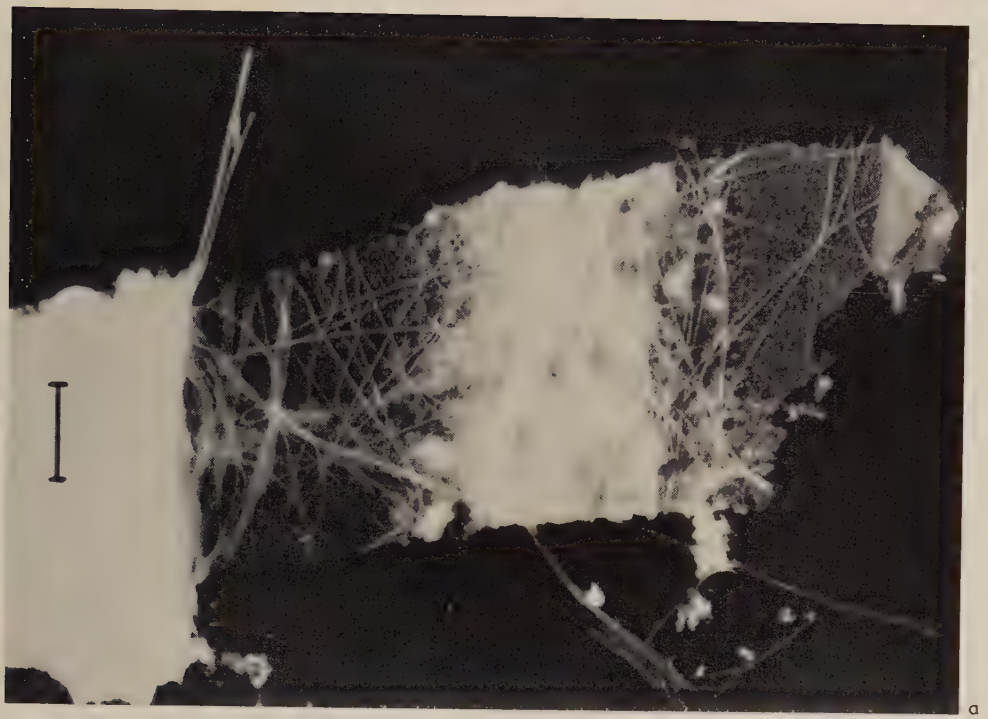
c



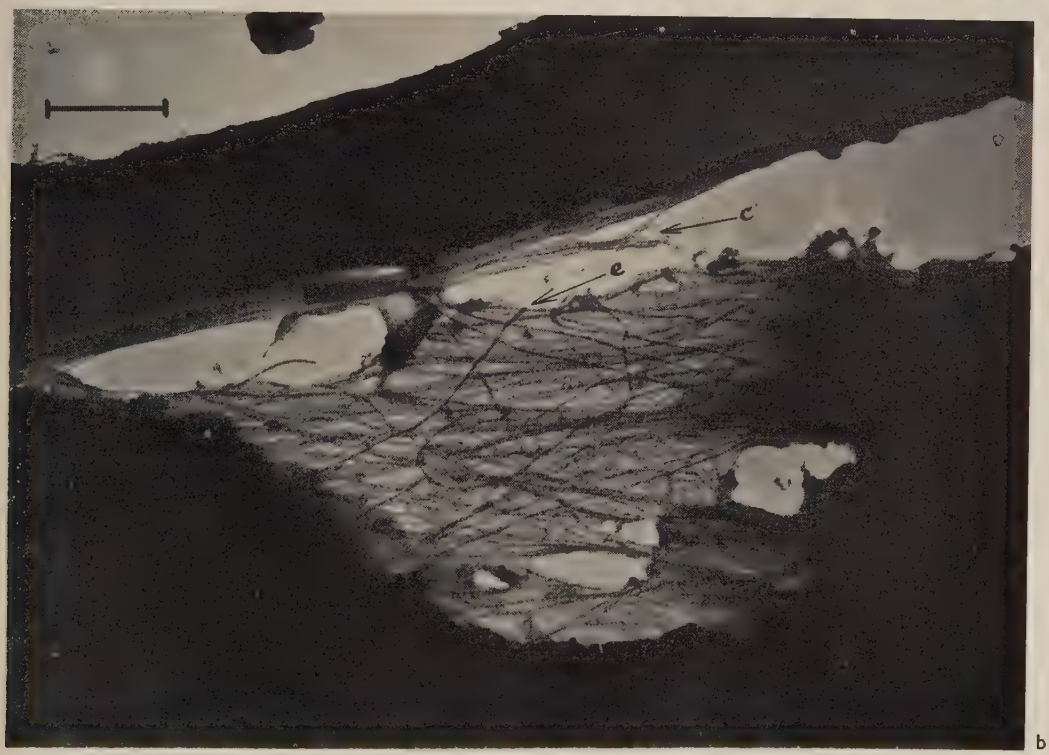
a



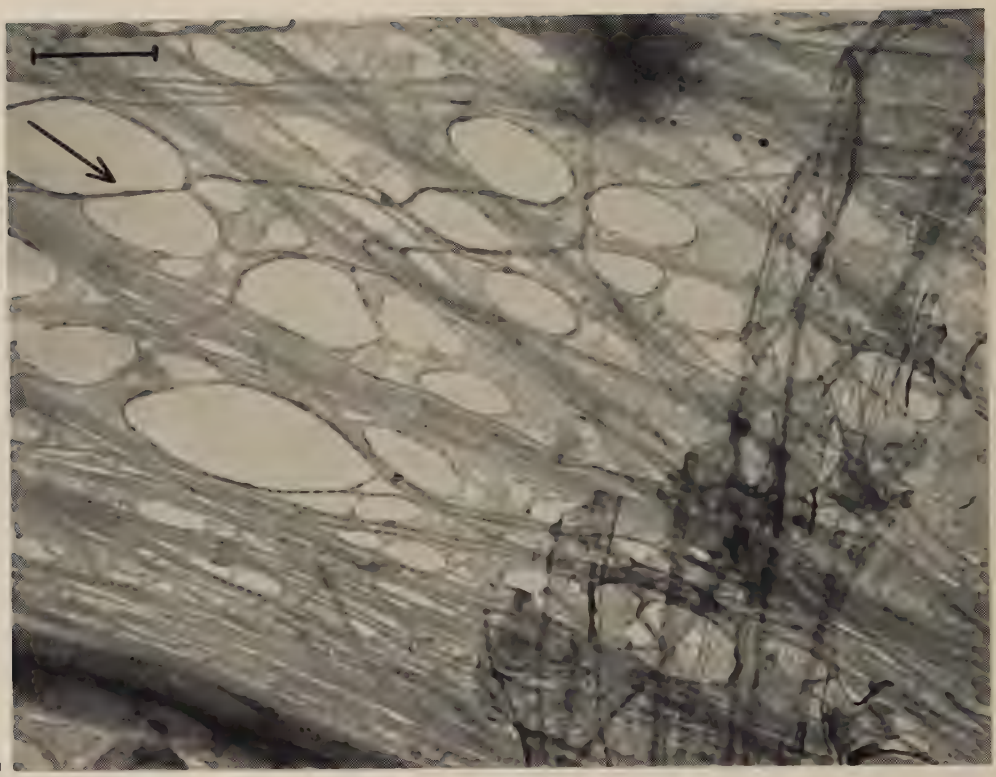
b



a



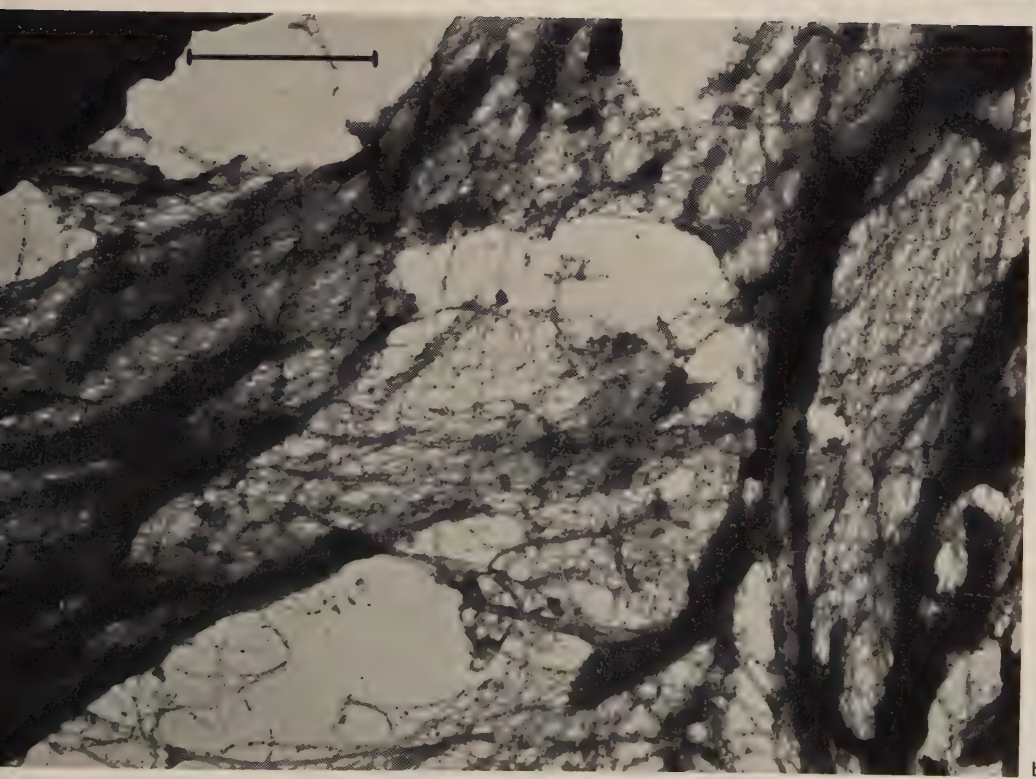
b



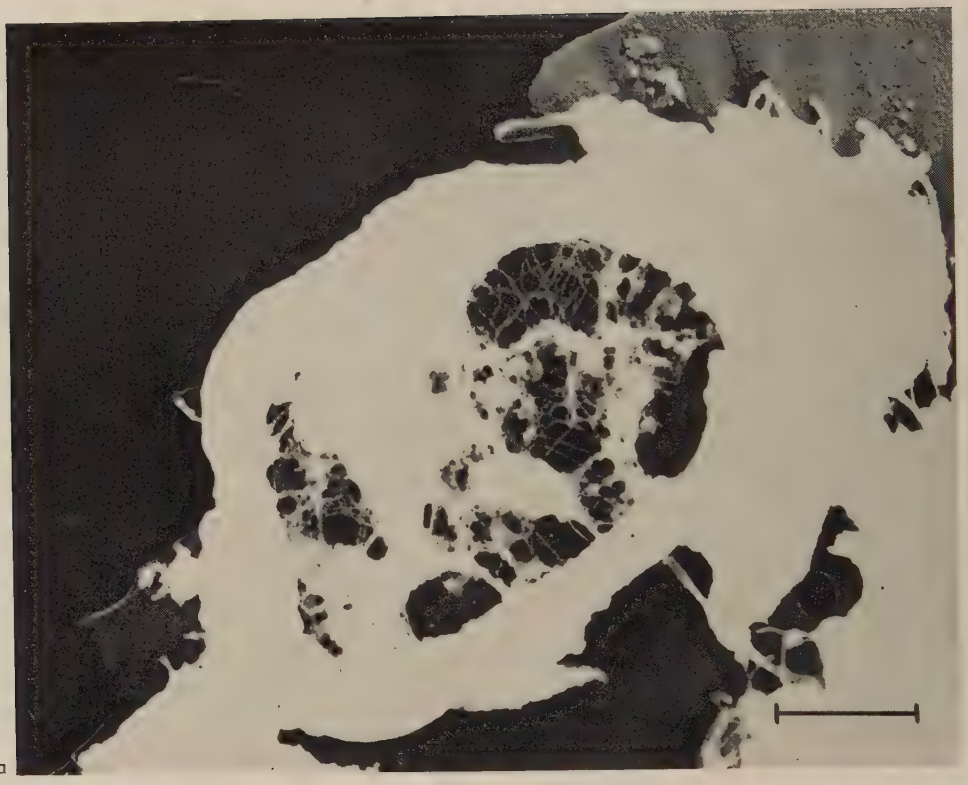
a

b

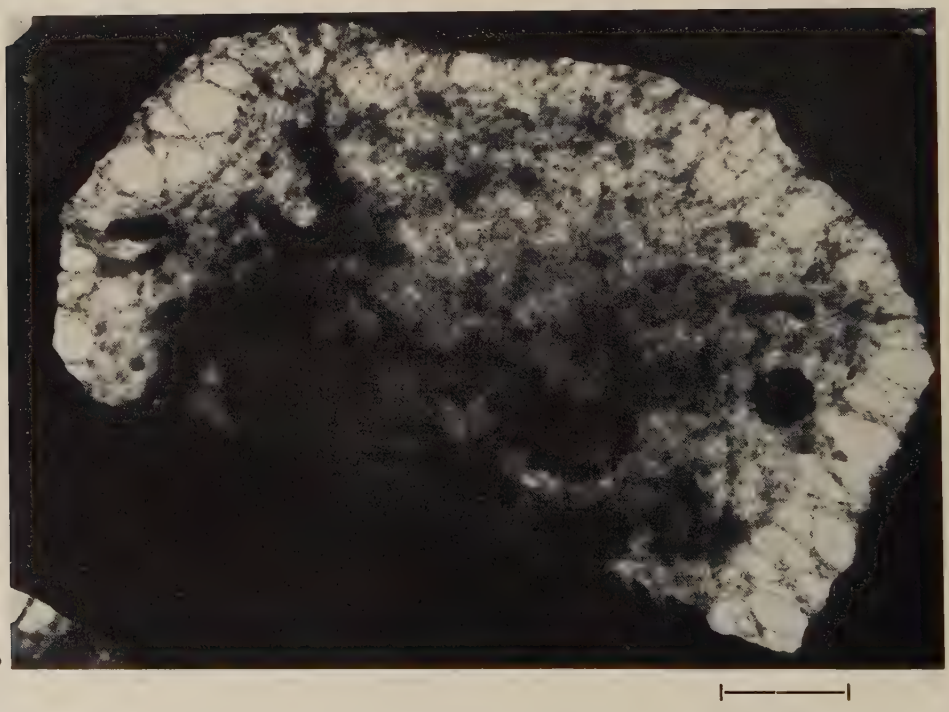
a



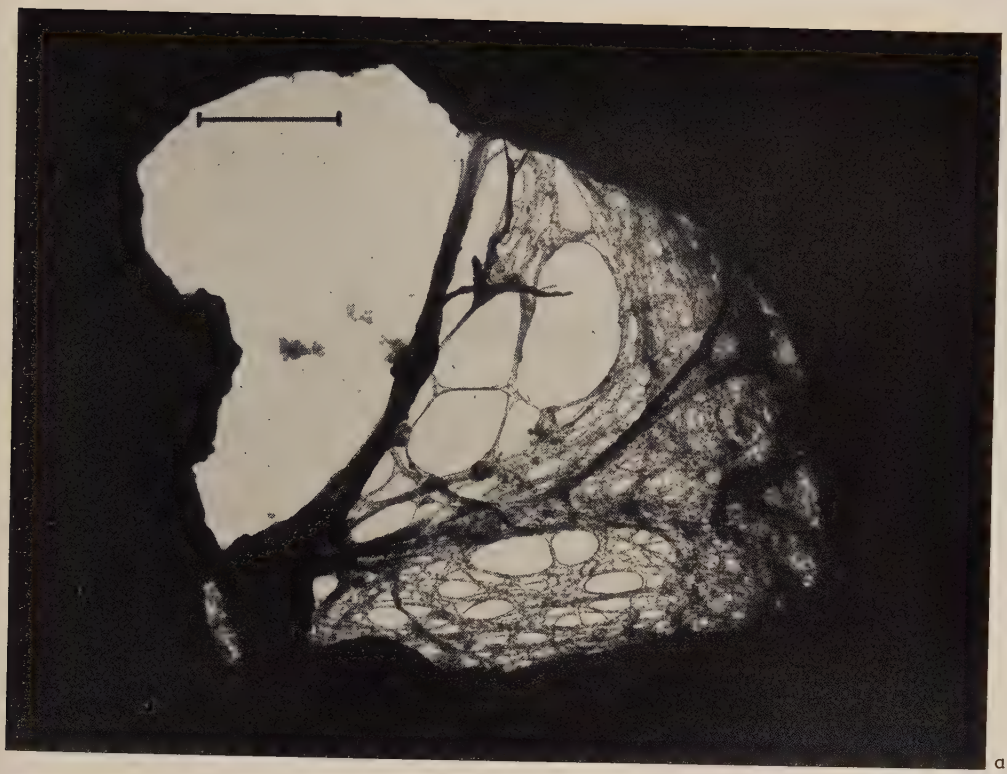
b



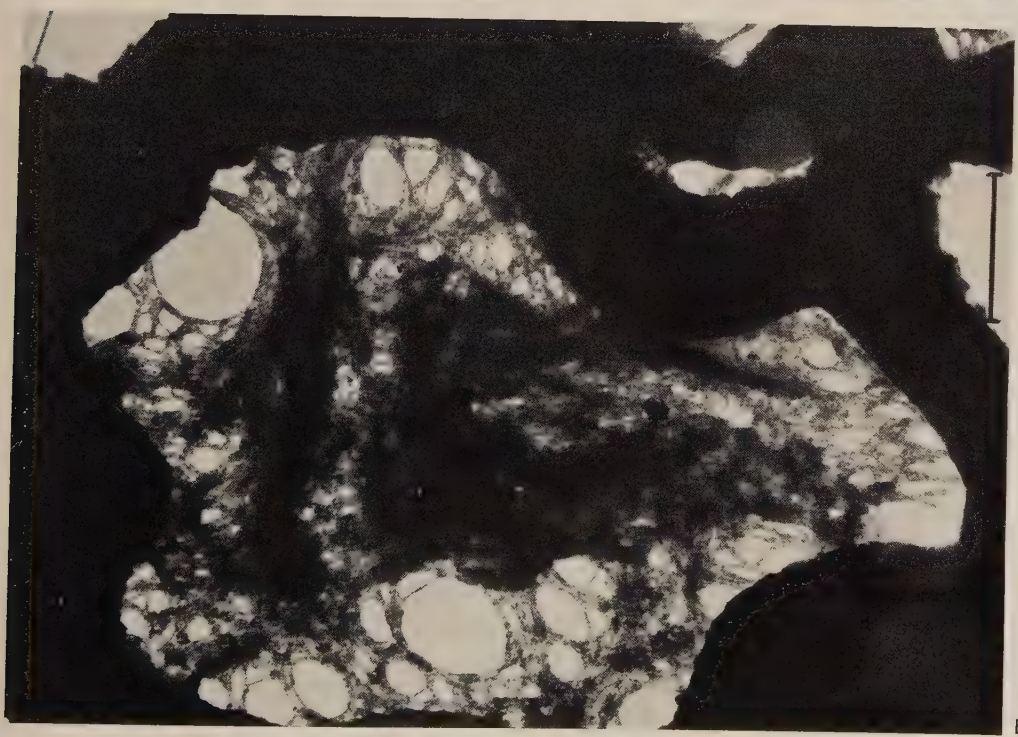
a



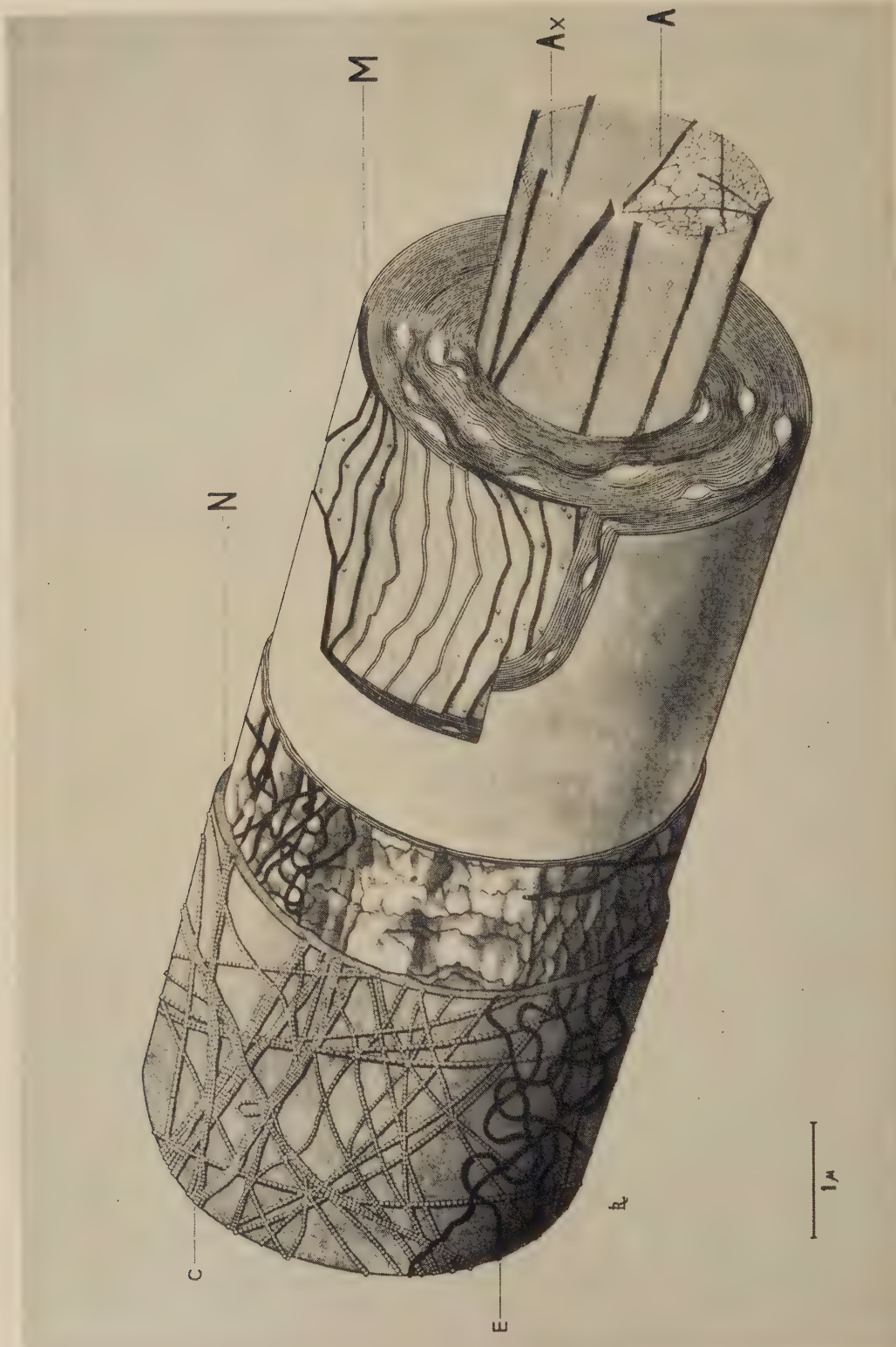
b



a



b


A
Ax
M
N
C
E
1μ

THE STRUCTURE OF PLANT SLIMES

KURT MÜHLETHALER[1]

Laboratory of Physical Biology, Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Maryland

Received December 10, 1949

INTRODUCTION

Occurrence of slimes

Slimes are very widespread among plants. They are found as well with bacteria, algae, fungi, lichens, mosses and ferns as with the gymnosperms and angiosperms. Ordinarily only individual cells or groups of cells produce slime, rarely an entire tissue. It is produced by the middle lamellae of cells of *Laminaria* (sea weed) and Carrageen (Irish moss), for example. Other plants have real slime glands which can be developed in the form of hairs, but the great majority of plant slimes are membranous. Nearly always the latter are laid down in place of the secondary walls of the cells. Such gelatinous thickened layers can become so thick that the original lumen of the cells is reduced to small slits. For the most part such deposits are found in the epidermal cells of seeds. In some cases, as with the seeds of quince (*Cydonia*), only the outer walls are thickened, while with others, such as flax (*Linum*) and hemp (*Cannabis*) seed, thickening involves both the inner and outer walls. The epidermal cells are then separated from the environment by a thin cuticle. When the seeds are moistened, the slimy contents of the cells swell and the outer wall ruptures. These slime membranes are laid down as such at the beginning and only rarely develop by metamorphosis from already existing secondary walls; according to Klebs (3), slime helps fix the seeds in the earth and serves for the storage of water.

Classification and chemistry

Several attempts have been made to classify slimes (4, 8). This can be done either according to their origin or their chemistry. Under the optical

[1] Special fellow of the National Institutes of Health. Permanent address: Pflanzenphysiologisches Institut der Eidg. Techn. Hochschule, Zürich, Switzerland.

microscope the different slimes appear so homogenous that no distinction can be drawn between them. Cellulosic and "true" slimes can, however, be distinguished on the basis of their different reactions to iodine-containing reagents. The true slimes are colored yellow by iodosulfuric acid, zinc chloroiodide and potassium iodide, and yield mucic as well as oxalic acid on oxidation with nitric acid. They are insoluble in copper-ammonia reagent. To this group belong flax (*Linum usitatissimum*), plantain (*Plantago psyllium*), etc. The cellulosic slimes are colored blue by iodosulfuric acid and zinc chloroiodide and yield only oxalic acid on oxidation. The slimes of quince (*Cydonia*) and sage (*Salvia*) belong to this group.

The slimes as a group are very hard to analyze chemically since they are not single substances and have a composition that is constantly varying. This prevents significant generalizations from results with individual slimes. It has, however, been established that they are all polyuronides and have aldobionic acid as the most important constituent. Their hydrolysis yields various sugars, such as xylose, galactose, glucose, arabinose, etc. As already indicated many contain cellulose. As a typical example quince slime (7) has the composition:

Uronic acids 28 per cent, pentoses 33 per cent, cellulose 33 per cent, arabinose 2 per cent, ash 2 per cent. For cress (*Lepidium*) a cellulosic content (2) of 18 per cent has been found. It is astonishing that the slime with such a high cellulosic content should be so readily soluble in water; and this emphasizes the question of how its cellulose is distributed. Heretofore, it has been the general opinion that solubility was due to the existence of a molecular complex of polyuronic acids and cellulose (7). For this the name muco-cellulose has been used. It would obviously be of the greatest interest, both scientifically and for the fiber industry, to demonstrate such a soluble cellulose-complex.

Purpose of investigation

In this study electron micrographs have been made of a number of slimes. This has been done to see what their fine structure may be, and especially to determine what may be the relation between their cellulosic and non-cellulosic components.

EXPERIMENTAL

The seeds to be studied were first swelled in moderately warm water. During this period the entire surface became covered with a layer of slime that appears under crossed nicols as that of a doubly refracting spherite

with $n\gamma$ running radially. To make a preparation a swollen seed was picked up with tweezers and carefully drawn over the collodion-covered screen so as to leave behind a delicate film of the slime. After drying, such a preparation was shadowed in the usual fashion (9) with chromium or palladium and examined in an RCA Type EMU electron microscope.

The cellulose component of slimes is most easily obtained by hydrolysis with 2.5 per cent sulfuric acid. After refluxing for about ten hours it appears as a brownish precipitate which is no longer water soluble. Preparations of these purified cellulose residues, as well as the fresh preparations outlined above, have been studied to throw light on the distribution of the cellulose and polyuronic acids.

RESULTS

Quince slime (Cydonia vulgaris)

As Fig. 1 indicates, the electron microscope shows quince slime to consist of filamentous strands distributed in a structureless menstruum. In both form and size these strands are identical with microfibrils of cellulose found in cell wall (5). These fibrils are not, however, here interwoven to form a sheet but are separately distributed throughout the slimy ground substance. This is clear if one realizes that the deposit in the dried preparation is in reality a projection of a thick layer of slime upon a plane. In the native state the cellulose fibrils are probably far less closely intertwined than Fig. 1 suggests; otherwise strong swelling could scarcely occur.

Cress slime (Lepidium sativum)

The cellulose of cress slime, too, is in the form of microfibrils. In contrast to what was seen with quince slime, however, there is a definite order in the arrangement of these fibrils. As Fig. 2 shows, they lie more or less parallel to one another in spite of disorientation brought about by the swelling (Fig. 3). This structure was foreshadowed by optical microscopic observations (8) which indicated that most membranous slimes are deposited on the cell wall as layers one lying on top of another. This is the same kind of layering that characterizes the secondary walls of plant fibers. In secondary walls each layer of microfibrils has a direction turned with respect to the layers above and below it. Fig. 2 shows clearly that a similar

cross-layered system of cellulose fibrils exists in this slime. Here the microfibrils of succeeding layers are turned through an angle of ca 60° with respect to one another. This layering strongly suggests that the deposition of the slime lamellae proceeds along the same general lines as the formation of normal secondary wall.

The figures show that cellulose microfibrils can be better seen in cress slime than in most cell walls where they are very closely clumped together. Their length is very variable and can amount to five micra or more. It has been especially interesting to examine their ends to see if there is any evidence that they taper down to still smaller fibrils. These ends have, however, always been smooth and uniform, with no indication of brush formation.

Linseed slime (*Linum usitatissimum*)

The slime of flax seeds, as already indicated, is a "true" slime which does not react chemically for cellulose. The electron microscope, however, reveals the presence of many cellulose-like particles within the amorphous ground substance. As Fig. 4 shows, these do not have the form of the long familiar strands, but are short spindle-like fibrous aggregates. The nonunifrom particles constituting them have lengths of not more than 0.1 micron. Often these particles appear as bundles of a few still finer needles. The diameters of these, the smallest units yet seen, seem to be between 60 and 80 A. According to their size they could be identical with the micelles of cellulose predicated from X-ray data. Masses of such particles, without longer fibrils, are often found (Fig. 5). If the amorphous, interstitial polyuronide is hydrolyzed, these particles show a tendency to join one another to form chains. For Fig. 6 a sample was taken before final solution of the polyuronide, but such chains can already be observed. When the polyuronide is completely hydrolyzed, the residue has a definitely fibrous structure (Fig. 7). Then single, separately distributed particles are no longer to be seen for all have united into the fibrous strands. The orientation within these fibrils is incomplete, and their surfaces are therefore irregular. Ordinarily they have lengths of 1–2 micra, but isolated strands have been seen up to five micra long.

Rosenbergia slime (*Cobaea scandens*)

Bands of cellulose visible in the optical microscope have earlier been found in the epidermal slime of the seeds of *Cobaea* (1). They appeared

as solid threads up to five centimeters long with an elliptical cross section and diameters of only a few micra. During ripening of the seeds, the strands develop within the protoplasmic coating and are coiled up spirally at that time. Their synthesis proceeds so rapidly that it has not been possible to follow exactly the process.

Electron microscopic examination has shown that a large number of microfibrils occur along with these large cellulose bands. As can be seen from Fig. 8 the microfibrils agree in form and size with those in quince and cress slimes. The bands are parallel associations of such microfibrils and it is therefore not surprising that they should be formed very rapidly since this process only need involve an assembly of units already present. Fig. 9 shows a similar partially finished strand of *Lepidium* slime.

Tragacanth slime

This slime has a chemical composition different from the foregoing. It contains the insoluble gum, bassorin, as well as a kind of water soluble gum, together with cellulose, starch and mineral constituents. It is not a membranous slime but is formed in the piths of various members of the genus *Astragalus*. The electron microscopic picture shows (Fig. 10) that here also cellulose is present as the usual microfibrils. Along with such fibrils there are always numerous ring-shaped particles about a micron in diameter (Fig. 11). Both the function and mode of formation of these particles remain doubtful. They are present in large numbers in the cellular protoplasm and belong to the class of objects ordinarily designated under the term microsome. Similar bodies are not seen in the other slimes even though their cellular protoplasm has been carried along with the emerging slime; and it would therefore seem that these particles are characteristic of Tragacanth slime.

Slime from the root tips of corn (maize)

When corn sprouts in a moist room, droplets of a slimy coating form on the root tips. If a thin film from a root tip is transferred to a specimen screen, it is seen that the ground substance is not amorphous but is composed of exceptionally small particles (Fig. 12). Since this slime gives no reaction with cellulose reagents it could not, however, be decided if these are particles of cellulose.

DISCUSSION

Our results do not confirm the previous opinion (7) that molecular associations of cellulose and uronic acids exist. Instead the cellulose in slimes appears as the normal microfibrils and there are no morphological grounds for retaining the term "muco-cellulose." The short particles in flax slime, if they are of cellulose, conform to our previous results with B. xylinum (6) which indicated that the synthesis of fibrils proceeds step-wise. It should be noted, however, that transitional forms to the long cellulose fibrils have not been seen in this or other slimes; instead all such microfibrils appear as already finished.

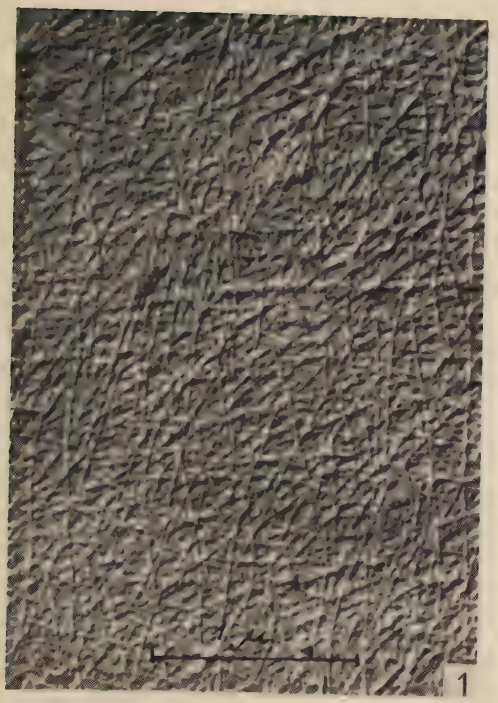
In all these slimes cellulose strands are carried along with their enveloping polyuronides during swelling and can therefore remain suspended in the more or less dilute suspension they provide. When the polyuronides are dissolved away in the laboratory, then the cellulose fibrils associate together and precipitate out as an insoluble residue.

This investigation was carried out in the laboratory of Dr. Ralph W. G. Wyckoff, to whom the writer is grateful for many helpful discussions.

SUMMARY

Several slimes have been investigated with the electron microscope. With one exception (*Linum*) their cellulose has the same microfibrillar form that has been found in cell walls. These microfibrils are separately suspended in the polyuronic acids as an amorphous ground substance; they are increasingly dispersed as the swelling proceeds.

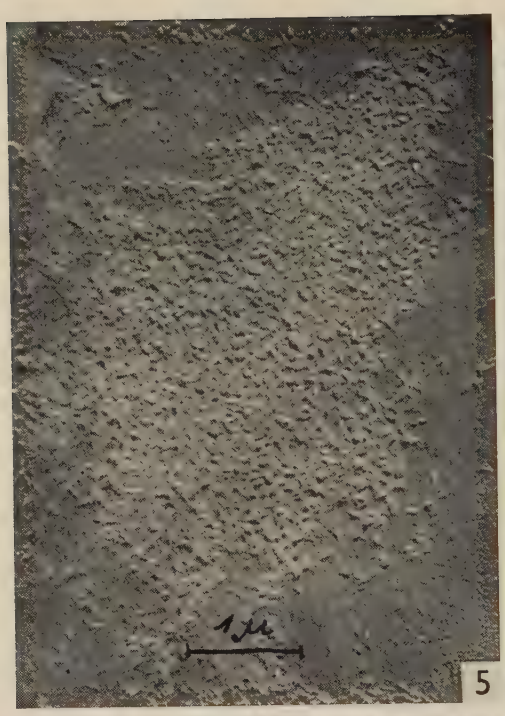
Short particles occur in linseed (*Linum*) slime. These show a marked tendency to become attached together in chains when the ground substance is dissolved away. This is a "true" slime which does not give the usual chemical tests for cellulose; it is therefore not clear if these short particles should be classed as cellulose; the previous supposition that mixed cellulose-polyuronide molecules exist is not confirmed by the electron microscopic observations.


1

2

3

4


1 µ
5

1 µ
6

1 µ
7

1 µ
8

1μ
9

10

1μ
11

1μ
12

REFERENCES

1. Ambronn, H., *Zsigmondy-Festschrift Koll. Z.*, **36**, 119 (1925).
2. Bailey, K., *Biochem. J.*, **29**, 2477, 2483 (1935).
3. Klebs, G., *Unters. a. d. Bot. Inst. Tübingen*, **1**, 581 (1885).
4. Mangin, L., *Bull. Soc. Bot.*, **41**, 307 (1894).
5. Mühlethaler, K., *Biochim. Biophys. Acta*, **3**, 15 (1949).
6. —— *Biochim. Biophys. Acta*, **3**, 527 (1949).
7. Renfrew, A. S., and Cretcher, L. H., *J. Biol. Chem.*, **97**, 503 (1932).
8. Tschirch, A., *Angewandte Pflanzenanatomie*, Wien und Leipzig, 1889.
9. Williams, R. C., and Wyckoff, R. W. G., *J. Applied Phys.*, **17**, 23 (1946).

Fig. 1. Quince seed mucilage (*Cydonia vulgaris*). The cellulose is in the form of microfibrils embedded in an amorphous ground mass of polyuronides. Magnification = 27,000 X.
Fig. 2. Cress seed mucilage (*Lepidium sativum*). The microfibrils are in more or less parallel array. Layers lying one above another are turned through ca 60°. Magnification = 22,500 X.
Fig. 3. Cress seed mucilage (*Lepidium sativum*). The microfibrils have become disoriented by the swelling. Magnification = 15,000 X.
Fig. 4. Linseed mucilage (*Linum usitatissimum*). Note the short fibrous bundles. No microfibrils of ordinary cellulose are present. Magnification = 18,000 X.
Fig. 5. Linseed mucilage (*Linum usitatissimum*). A large mass of the short particles. Magnification = 15,000 X.
Fig. 6. Linseed mucilage (*Linum usitatissimum*). On removal of the ground substance these short particles show a tendency to build fibrils. Magnification = 18,000 X.
Fig. 7. Linseed mucilage. Here all polyuronides have been hydrolyzed away. All short fibers have become part of a fibrillar network. Magnification = 25,000 X.
Fig. 8. Slime from the epidermis of a seed of *Cobaea*. The cellulose has its usual microfibrillar form. Magnification = 25,000 X.
Fig. 9. Cress seed mucilage (*Lepidium sativum*). Bands visible in the optical microscope arise from a piling together of normal microfibrils of cellulose. Magnification = 19,000 X.
Fig. 10. Tragacanth mucilage. The microfibrils here are normal. Magnification = 19,500 X.
Fig. 11. Tragacanth mucilage. This slime is full of these ringlike bodies. Magnification = 14,000 X.
Fig. 12. Slime from the root tips of corn. Magnification = 18,000 X.

BRIEF NOTES

ACTION OF FICIN ON SEA URCHIN EGGS

AA. BOHUS JENSEN

Biological Institute, Carlsberg Foundation, Copenhagen

Received January 23, 1950

It is well known that proteolytic enzymes do not digest living cells or organisms (6, 7, 8) with the possible exception that ficin digests living intestinal worms (9).

Crystalline trypsin easily destroys the vitelline membrane covering the cytoplasmic surface of the unfertilized sea urchin egg (10). Above a certain concentration it also inhibits the fertilization of the eggs (10, 13, and unpublished investigations of the present author). Interesting changes were observed after treating the eggs of the annelid Pomatoceros triqueter with trypsin (5).

An activating effect of commercial preparations of trypsin on eggs of several sea urchin species has been reported (1, 12) as well as on eggs of Fucus and Hormosira (2, 3) whereas crystalline trypsin did not activate the eggs of *Psammechinus miliaris*, *Ps. microtuberculatus*, *Sphaerechinus granularis* and *Paracentrotus lividus* (1, and unpublished observations).

The activation brought about by commercial trypsin is therefore probably due to a component other than trypsin. For a certain activating effect of crystalline trypsin on the egg of *Echinocardium cordatum* see (10).

The object of the present investigation was to see whether ficin activates the unfertilized sea urchin egg, or influences its fertilizability.

Psammechinus miliaris Gmelin from the neighbourhood of the Kristineberg Zoological Station, Sweden, served as material. The experiments were carried out at 22–25° C. The jelly coat was not generally removed from the eggs.

When unfertilized eggs were subjected to an 0.2–0.4 per cent solution of dialysed ficin preparation (Merck. a. Co., USA) in sea water at pH 8.2 for 1 to 4 hours they showed nuclear activation followed by irregular cleavage of the cells.

In 25 different experiments in which the eggs were kept in the ficin solution during the whole observation time only 10 to 80 per cent of the eggs were activated, although 100 per cent fertilization was found in the control samples.

In some experiments the eggs were treated with an 0.4 per cent solution of ficin for 1 hour, after which they were washed several times before being transferred to sea water. The percentage of activation here was 10 to 50.

The nuclei of these activated eggs moved to the centre of the eggs, the nuclear membrane was dissolved and in several cases the formation of a monaster was also observed.

A nuclear division frequently occurred together with a more or less irregular cytoplasmic cleavage into 3 to 7 cells, in several cases of almost equal size and each usually containing one nucleus. Further development did not occur.

No digestion of the eggs was observed; (see also 7). After treatment of the eggs in an 0.2 per cent ficin solution for 20 to 60 minutes followed by insemination in the ficin solution, only about 10 per cent of the eggs were fertilized. Ficin thus has a strong inhibitory effect on fertilization.

In the cases where egg fertilization occurred after ficin treatment, no fertilization membrane was formed owing to enzymatic destruction of the vitelline membrane. A hyaline layer appeared, however, even in an 0.1–0.2 per cent ficin solution, and was found to be resistant to the enzyme. The cleavage cells therefore did not fall apart; (see also 7).

According to one report (11), a commercial trypsin (Merck, Darmstadt) causes a destruction of the hyaline layer in *Psammechinus* eggs, resulting in a separation of the cleavage cells.

The present author did, however, notice that the hyaline layer in eggs of *Sphaerechinus granularis* and *Paracentrotus lividus* are able to resist the action of crystalline trypsin for hours.

It is of interest in this connection that Monné (4) found carbonhydrates to be important constituents of the hyaline layer.

Ficin evidently acts on the surface of the egg in much the same way as trypsin does, i.e. it dissolves the vitelline membrane and, above a certain concentration, it inhibits fertilization. It is so far not settled whether the activating effect described above depends on the enzyme ficin or on some impurity in the sample which may be similar to the activating substance of commercial trypsin preparations.

The author is indebted to Prof. J. Runnström, Stockholm, for his interest in this investigation, to Merck a. C., Rahway, N. J., USA, for the ficin preparation, and to "Wenner-Grenska Samfundet", Stockholm, for a grant in aid of this work.

REFERENCES

1. Bohus Jensen, Aa., *Nature*, **162**, 931 (1948).
2. Levring, T., *Medd. Göteborgs Bot. Trädgård*, **17**, 97 (1947).
3. —— *Physiol. Plantarum*, **2**, 45 (1949).
4. Monné, L., *Personal communication.*
5. Monroy, A., *Arkiv Zool.*, **40** *A*, No. 21 (1948).
6. Northrop, J. H., *J. Gen. Physiol.*, **9**, 497 (1926).
7. —— *J. Gen. Physiol.*, **30**, 375 (1947).
8. Oppenheimer, C., *Die Fermente und ihre Wirkungen. Suppl.* 1–2 (1936–39).
9. Robbins, B. H., *J. Biol. Chem.*, **87**, 251 (1930).
10. Runnström, J., *Ark. f. Zool.*,, **40** *A*, No. 17 (1948).
11. Runnström, J., Monné, L., and Broman, L., *Ark. f. Zool.*, **35***A*, No. 3 (1943).
12. Runnström, J., Monné, L., and Wicklund, E., *J. Coll. Sci.*, **1**, 421 (1946).
13. Wicklund, E., *Ark. f. Zool.*, **1**, No. 1 (1949).

PHOSPHORUS UPTAKE BY BACTHERIOPHAGE AND ULTRAVIOLET IRRADIATED BACTERIA

LOUIS W. LABAW, VERNON M. MOSLEY and RALPH W. G. WYCKOFF

Laboratory of Physical Biology, Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Maryland

Received March 11, 1950

Cells of E. coli, "killed" by exposure to ultraviolet light (1), can still be lysed by bacteriophage with the usual yield of new bacteriophage particles. Studies of radiophosphorus uptake by such systems involving irradiated bacteria is proving to be a source of especially interesting results.

Cultures exposed to a dose of unfiltered radiation from a quartzmercury lamp sufficient to reduce the developable colony count onehundred-thousand fold exhibit a steadily diminishing uptake of P^{32} (curve 2 of Fig. 1) which is at all times far less than that of a non-irradiated bacterial culture (2). If enough of a Berkefeld filtered broth suspension of bacteriophage is prompty added to such irradiated suspensions of E. coli to infect all susceptible bacteria (ca 5 bacteriophage particles per viable organism in a parallel unirradiated control), a P^{32} uptake occurs which is several times greater (curve 1) than that by the irradiated bacteria alone. This enhanced phosphorus uptake can be shown to be roughly proportional to the yield of new bacteriophage. Such demonstrable stimulation of the phosphorus anabolism in an irradiated bacteria-bacteriophage system is in striking contrast to conditions observed in a system composed of unirradiated bacteria and bacteriophage. We have found that when unirradiated bacteria are used any stimulation that may take place when bacteriophage is added is negligible compared to the phosphorus absorption of such bacteria growing in the absence of bacteriophage.

It would appear experimentally meaningless at this time to inquire if this increase is due to stimulation of the bacteria undergoing lysis or to an independent assimilation of phosphorus by the bacteriophage being formed. It is important in this connection, however, that we have found by ultracentrifugation most of the added phosphorus in this new bacteriophage. Our results thus presumably are recording the synthesis of desoxyribose nucleic acid from phosphorus of the medium, which Cohen (3) and others (4) have already described.

Results of detailed studies of phosphorus metabolism and of morphological changes as seen under the electron microscope in cultures of ultraviolet-irradiated E. coli and in systems composed of such bacteria plus bacteriophage will be published later.

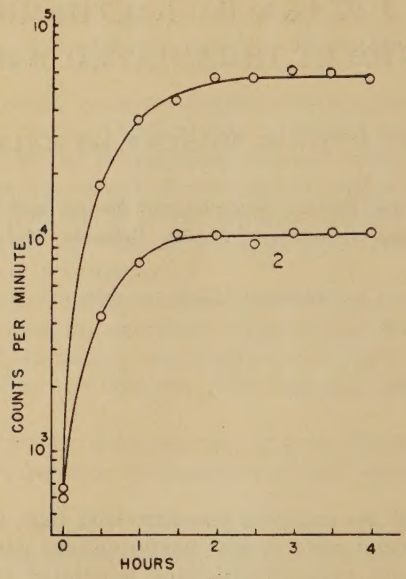


Fig. 1. Radioactive phosphorus taken up as a function of time by 5×10^9 ultraviolet irradiated E. coli (curve 2) and that taken up by similar coli infected with T_2 bacteriophage (curve 1). The bacterial concentration was 10^8 E. coli per ml of tryptose broth containing enough $Na_2HP^{32}O_4$. to give a count of 10^5 counts per minute per ml.

REFERENCES

1. Anderson, T. F., *J. Bact.*, **56**, 403 (1948).
2. Labaw, L., Mosley, V. M., and Wyckoff, R. W. G., *J. Bact.* (1950).
3. Cohen, S. S., *J. Biol. Chem.*, **174**, 295 (1948).
4. Putnam, F. W., and Kozloff, L. M., *Science*, **108**, 386 (1948).